Systemic inflammation and remote organ injury following trauma require HMGB1

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Submitted 20 April 2007; accepted in final form 25 July 2007

TRAUMATIC INJURY REMAINS THE leading cause of death in the United States for individuals under the age of 44 years (11). Injuries involving the bone and soft tissues represent a major cause of morbidity and mortality in those sustaining trauma. More than 90% of patients with multiple injuries suffer one or more long bone fractures (7). Extensive soft-tissue injury and bone fracture are significant contributors to the initial systemic inflammatory response in patients with multiple injuries. Systemic inflammation can also lead to organ dysfunction remote from the site of traumatic injury (41).

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
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cyanogen bromide-activated Sepharose beads following standard procedures. Neutralizing activity of anti-HMGBl was confirmed in HMGBl-stimulated macrophage cultures by assay of TNF release. In the presence of anti-HMGBl antibody, neutralizing was defined as inhibiting >80% of HMGBl-induced TNF release.

Animals. Mice used in the experimental protocols were housed in accordance with University of Pittsburgh and National Institutes of Health (NIH) animal care guidelines in specific pathogen-free conditions. The animals were maintained in the University of Pittsburgh Animal Research Center with a 12:12-h light-dark cycle and had free access to standard laboratory feed and water. Male C3H/HeOuJ (TLR4-WT) and C3H/HeJ (TLR4-Mu) mice (Jackson Laboratories, Bar Harbor, ME) were 8–12 wk old and weighed 20–30 g in these experiments. All animals were fasted for ~12 h prior to experimental manipulation and were acclimatized for 7 days prior to being studied.

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 and Care Committee of the University of Pittsburgh. Briefly, animals were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg) and inhaled isoflurane (Abbott Labs, Chicago, IL). With the use of sterile technique, a left groin exploration was performed, and the left femoral artery was cannulated with tapered polyethylene (PE)-10 tubing and connected to a blood pressure transducer (Micro-Med, Tustin, CA) for continuous mean arterial pressure (MAP) monitoring for the duration of the experiment (1 or 6 h). Bilateral, closed, midshaft femur fracture was then performed by using two hemostats applied to the hindlimb region. Immediately postfracture, mice received 600 μg of either anti-HMGBl antibody or nonimmune control IgG (Sigma-Aldrich) via intraperitoneal injection. MAP was maintained above 60 mmHg throughout the experiment with the administration of lactated Ringers solution (Baxter, Deerfield, IL) through the femoral cannula as needed in 0.1-ml boluses. This procedure served to ensure that the animals were not in a state of circulatory shock. According to the manufacturer, the endotoxin content of the lactated Ringers solution utilized was 0.008 EU/ml. Sham-operated mice underwent anesthesia and femoral cannulation only. All mice were reanesthetized with intraperitoneal pentobarbital sodium (20 mg/kg) as necessary throughout the experiment. Baseline MAP, total anesthetic dosage, and volume of lactated Ringers administered did not differ between strains of mice or experimental groups (sham vs. fracture). At the end of 1 or 6 h, mice were euthanized under inhalational anesthesia via cardiac puncture technique. Necropsy was performed to verify the presence of bilateral femur fractures and to ensure the absence of fracture site hematomas. Serum from postmortem 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-kB 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 HMGBl levels by ELISA. Mouse monoclonal anti-HMGBl (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 HMGBl produced in Escherichia 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 + 4% BSA at room temperature for 1 h on a shaking platform to allow capture of the HMGBl. Plates were washed three times in PBST (PBS + 0.2% Tween-20) for 10 min each in 200 μl. The detection antibody is a polyclonal antibody raised in rabbits to the human HMGBl peptide sequence 5′-KPDAAKKGVVKAEKS-3′ and was incubated at 0.5 μg/ml for 1 h at room temperature in PBS + 4% BSA. The samples were then washed three times with PBST for 10 min each in 200 μl. Donkey anti-rabbit horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA) was used as the final step for detection of the rabbit anti-HMGBl and was incubated for 1 h at room temperature with shaking in a final volume of 50 μl in PBS + 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 benzidine 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). Serum concentrations were determined using the freeware ELISA analysis program Titiri, 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, Chicago, IL). For comparisons between C3H/HeOuJ and C3H/HeJ mice, the sample size was six animals per group.

RESULTS

Neutralizing antibodies to HMGBl decrease systemic inflammation 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 HMGBl contributes to systemic inflammation and end-organ injury after peripheral tissue injury, we administered neutralizing antibodies to HMGBl to C3H/HeOuJ (TLR4-WT) and C3H/HeJ (TLR4-Mu) mice subjected to bilateral femur fracture. Animals received either 600 μg of anti-HMGBl or irrelevant IgG immediately after injury. After 6 h, the animals were killed and their sera analyzed for IL-6 and IL-10 levels. Consistent with our previous reports, TLR4-WT animals subjected to fracture and receiving control IgG demonstrated significant increases in systemic IL-6 and IL-10 compared with sham-treated mice, whereas TLR4-Mu mice did not (Fig. 1, A and B) (18). In contrast, TLR4-WT mice treated with anti-HMGBl after fracture demonstrated minimal increases in IL-6 and IL-10, with levels approximating those seen in TLR4-Mu animals. These data suggest a key role for HMGBl in the initiation of the systemic inflammatory response to trauma. In addition, these data confirm the requirement for TLR4 in the systemic inflammatory response to traumatic injury (18).

Neutralizing antibodies to HMGBl decrease hepatic inflammation and injury following bilateral femur fracture. We previously have shown that peripheral tissue injury results in inflammatory signaling in the liver by a TLR4-dependent mechanism (18). In these experiments, we investigated the role
of HMGB1 in fracture-induced end-organ inflammation and injury by assessing both ALT levels and hepatic NF-κB activation in animals subjected to femur fracture and subsequently treated with either anti-HMGB1 or nonimmune IgG. TLR4-WT mice subjected to femur fracture and treated with nonimmune IgG demonstrated significantly increased ALT levels compared with TLR4-Mu mice subjected to fracture (Fig. 2A). TLR4-WT mice receiving anti-HMGB1 prior to fracture (Fig. 2A) or anti-HMGB1 after fracture (Fig. 2B) demonstrated decreased hepatic NF-κB activation compared with TLR4-WT mice receiving nonimmune IgG or anti-HMGB1 after fracture. These data support a novel role for 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 activation was significantly reduced in mice treated with anti-HMGB1 before fracture (Fig. 2B).

Fig. 1. Neutralizing antibodies to high-mobility group box 1 (HMGB1) decreased systemic inflammation after bilateral femur fracture. Mice that underwent either sham procedure or bilateral femur fracture were treated with anti-HMGB1 antibody (600 μg) or an equivalent dose of control antibody immediately after injury. Serum IL-6 (A) and IL-10 (B) levels were analyzed for analysis of systemic inflammation. Toll-like receptor 4 (TLR4) wild-type (WT) mice treated with control IgG after fracture demonstrated significantly increased levels of IL-6 and IL-10 compared with TLR4-mutant (TLR4-Mu) mice (*P < 0.05). TLR4-WT mice treated with anti-HMGB1 after fracture demonstrated levels of IL-6 and IL-10 similar to those seen in TLR4-Mu mice. Data represent means ± SE, n = 6 mice per group. Fx, bilateral femur fracture.

Fig. 2. Neutralizing antibodies to HMGB1 decreased hepatic inflammation and injury after bilateral femur fracture. Mice that underwent either sham procedure or bilateral femur fracture were treated with anti-HMGB1 antibody (600 μg) or an equivalent dose of control antibody immediately after injury. Serum alanine aminotransferase (ALT; A) levels were analyzed for analysis of hepatocellular injury. TLR4-WT mice treated with control IgG after fracture demonstrated significantly increased ALT levels compared with TLR4-Mu mice (*P < 0.05). TLR4-WT mice treated with anti-HMGB1 after fracture demonstrated ALT levels similar to those seen in TLR4-Mu mice. Data represent means ± SE, n = 6 mice per group. Fx, bilateral femur fracture.

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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-WT 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.
Inflammatory trigger associated with femur fracture might not involve DNA, and phospholipids, it seems plausible to suggest that the HMGB1 avidly interacts with numerous other substances, leading to cytokine-like activity (37). Studies using TLR4 chimeric mice have shown 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-Mu 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 tissues 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, Vallabhaneni R, Vodovoz 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, Vodovoz 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 WT and TLR4 Mu 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).

ACKNOWLEDGMENTS
The authors acknowledge the technical assistance of Hong Liao, Laurn Kohut, and Derek Barclay.

GRANTS
This work was supported by National Institutes of Health Grants GM-53789-08 (to T. R. Billiar, Y. Vodovoz, M. P. Fink), CA-101944-01 (M. T. Lotze), and GM-65583-01 (D. J. Hackam). K. P. Mollen, J. M. Prince, D. J. Kaczorowski and R. Vallabhaneni are recipients of American College of Surgeons Resident Research Scholarships.

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

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