High Mobility Group Box–1 as a Therapeutic Target Downstream of Tumor Necrosis Factor

Christopher J. Czura, Huan Yang, and Kevin J. Tracey
Laboratory of Biomedical Science, North Shore–Long Island Jewish Research Institute, Manhasset, New York

The discovery of tumor necrosis factor (TNF) as a mediator of lethal sepsis and the effectiveness of anti-TNF monoclonal antibodies in experimental studies created widespread interest in the possibility that cytokine responses might be manipulated to therapeutic advantage [3]. Anti-TNF antibodies are approved for the treatment of rheumatoid arthritis and inflammatory bowel disease, but not for sepsis. A major reason for inefficacy in sepsis is that proinflammatory cytokines are released early in the septic response, usually within minutes. The kinetics of this response therefore hinder development of anti-cytokine strategies, because cytokine responses normalize before therapy can be rationally initiated. For example, anti-TNF antibodies provide significant protection against endotoxin lethality and gram-negative bacterial sepsis, but only when administered early; even a minimal delay impairs success [3–5]. The early and transient activity of TNF and other early mediators limit the practicality of targeting these mediators in most cases of sepsis.

We initiated a program to identify other more accessible or “late” targets. Support for this approach came from 2 central observations. First, death from severe sepsis often occurs as a late phenomenon, and we began a search for putative “late” mediators that could be targeted after the onset of infection. We have now identified high mobility group box–1 (HMGB1) as a late mediator of endotoxemia and sepsis. HMGB1 is released by activated macrophages, induces the release of other proinflammatory mediators, and mediates lethality when overexpressed. Administration of anti-HMGB1 antibodies inhibit systemic inflammation, even in established cases, because HMGB1 activity is elevated at significantly later time points than TNF or interleukin-1. It will now be important to determine whether this wider window of activity can be translated into therapeutic advantage for human inflammatory disease.

Bloodborne bacterial infection can lead to severe sepsis, a lethal syndrome of organ failure and cardiovascular collapse with a mortality rate of 30%–50%. Approximately 750,000 cases of severe sepsis occur in the United States each year; at least 225,000 of these cases are fatal, accounting for 9.3% of all deaths in the United States annually and equaling the number of fatalities from myocardial infarction [1]. Activated protein C recently was shown in phase 3 trials to reduce the risk of death from severe sepsis and septic shock by 6.1% [2], but, despite this advance in intensive care therapy, efficacious treatments are still needed.

Informed consent was obtained from all patients or their parents/guardians, and human experimentation guidelines of the US Department of Health and Human Services and of the North Shore University Hospital Institutional Review Board were followed in the conduct of clinical research.

Financial support: National Institutes of Health (National Institute of General Medical Sciences).

Reprints or correspondence: Dr. Kevin J. Tracey, Laboratory of Biomedical Science, North Shore–Long Island Jewish Research Institute, 350 Community Dr., Manhasset, NY 11030 (kjtracey@sprynet.com).

The Journal of Infectious Diseases 2003; 187(Suppl 2):S391–6 © 2003 by the Infectious Diseases Society of America. All rights reserved. 0022-1899/2003/18712S-0014$15.00
endotoxemia often occurs hours or days after the early TNF response. Second, high doses of endotoxin are fatal to genetically engineered TNF knockout animals, also after a delay of hours or days. We reasoned that these observations might be reconciled by the appearance of a putative late-acting mediator of endotoxin lethality, distinct from TNF. Theoretically, therapeutic targeting of such a mediator might expand the window of opportunity for treatment of sepsis. Our study of this hypothesis resulted in identification of high-mobility group box–1 (HMGB1) as a late mediator of endotoxin lethality [6]. This ubiquitous, highly conserved DNA-binding protein was previously known to participate in DNA transcription, replication, and repair and in cell motility and neurite outgrowth [7–9]. It is now clear, however, that HMGB1 is also a cytokine when released into the extracellular milieu and that it participates in the development of lethality. Like other critically important cytokines, HMGB1 has multiple mediator activities, including stimulating the release of other cytokines, amplifying the development of acute lung injury, and causing inflammation [6, 10–12].

CLASSICAL FUNCTIONS OF HIGH MOBILITY GROUP PROTEINS

HMGB1 is a member of the superfamily of high mobility group proteins that was first isolated and characterized by Johns in the early 1960s and named for its electrophoretic mobility on polyacrylamide gels [13]. HMGB1 itself was discovered in the 1970s in calf thymus as an abundant chromosomal protein [14]. The high mobility group proteins are now classified in 3 groups on the basis of structure: the HMGB family, which is characterized by a functional domain known as the HMG “box,” the HMGN family, which contains a characteristic nucleosomal binding domain; and the HMGA family, which contains a unique “AT-hook” domain [15]. In eukaryotes, HMGB family members are among the most ubiquitous, abundant, and evolutionarily conserved proteins, with 80% aa sequence identity among higher eukaryotic species [16]. It is an abundant component of chromatin as each nucleus contains some 10 6 HMGB1 molecules.

Human HMGB1 has 219 residues in its primary amino acid sequence. Mature HMGB1 migrates as a 30-kDa protein, even though HMGB1 cDNA predicts a molecular mass of 25 kDa. The N-terminus is rich in positively charged lysine residues, whereas the highly conserved C-terminus (also termed the “acidic tail”) is rich in negatively charged aspartic and glutamic acid residues [13]. HMGB1 and other members of the HMGB family share a common structural motif known as the HMG box, an ~80-residue, L-shaped domain that mediates DNA binding [17]. The HMG boxes provide the structural requirements for DNA binding and recognition of distorted DNA structures such as 4-way junctions, cruciform DNA, and cisplatin-DNA adducts [18]. Each member of the HMG family contains ≥1 HMG box; HMGB1 has 2, 1 in the N-terminal domain and 1 in the central domain [19].

HMGB1 IS A CYTOKINE DOWNSTREAM OF TNF

Activities in vitro. HMGB1 was discovered after a search for a putative mediator of endotoxin lethality that exhibited delayed kinetics, compared with TNF. HMGB1 is secreted by macrophages, monocytes, and pituicytes after stimulation with endotoxin, interleukin (IL)–1, and TNF [6, 10]. Its release from macrophages is delayed some 12–16 h after stimulation with endotoxin. HMGB1 release is observed when cells are stimulated with as little as 5 ng/mL TNF or 10 ng/mL IL-1 [10]. Pulse-chase labeling with 35S-labeled methionine reveals that most of the HMGB1 released during the first 12 h after exposure to TNF comes from a pool of previously synthesized material [6]. During prolonged stimulation, the HMGB1 released into the extracellular milieu contains 35S, indicating the induction of new protein synthesis that is exported by an unknown mechanism [6]. Indeed, the regulation of HMGB1 synthesis in activated macrophages and the secretion pathways and mechanisms are unknown.

Like other cytokine mediators of endotoxemia (i.e., TNF and IL-1), HMGB1 activates proinflammatory cytokine release from human monocytes, including TNF, IL-1α, IL-1β, IL-1 receptor agonist, IL-6, IL-8, macrophage inflammatory protein (MIP)–1α, and MIP-1β, but not IL-10 or IL-12 [12]. Of importance, TNF release after stimulation with HMGB1 is significantly delayed, compared with lipopolysaccharide (LPS)–induced TNF release. HMGB1-induced up-regulation of TNF mRNA and protein is biphasic, peaking at 4 h and again at 10 h after addition of HMGB1. This biphasic response differs significantly from the monophasic, rapid kinetics of TNF typically observed after stimulation with LPS [12]. Digestion with trypsin completely abolishes HMGB1 cytokine-stimulating activity, indicating that the activity of HMGB1 is dependent on an intact protein [12]. HMGB1 can induce chemotaxis in rat smooth muscle cells in vitro [20] and can be passively released by damaged or necrotic cells [21]. In contrast, HMGB1 remains tightly associated with chromatin and is not released by apoptotic cells, indicating that release of HMGB1 accounts for whether cell death mediates inflammation (as in necrotic cell death) or not (as in apoptosis) [21].

In a recent structure function analysis, we found that cytokine activity of HMGB1 maps to the DNA-binding B box domain. Recombinant B box (aa residues 88–162) stimulates TNF release from a murine macrophage cell line (RAW 264.7) and is lethal when injected into BALB/c mice [22]. Both B box and full-length HMGB1 increase the permeability of cultured
human enterocytes [23]. A smaller domain (aa residues 129–138) is nontoxic, indicating that the cytokine activity of HMGB1 can be discretely localized [24]. Antibodies raised against the B box inhibit the activity of native HMGB1 [22], a finding that may have implications for rational design of HMGB1 inhibitors.

**In vivo endotoxemia and sepsis.** Serum HMGB1 levels are detectable only in very low levels (<2 ng/mL) in normal mice and humans. HMGB1 appears in the serum of mice 8 h after endotoxin infusion (25 ng of HMGB1/mL of serum), plateaus within 16 h (300 ng of HMGB1/mL serum), and remains elevated for ≥32 h. Similar, but somewhat delayed, HMGB1 serum kinetics are also observed in mice subjected to sepsis (authors’ unpublished data) induced by cecal ligation and puncture [25]. In response to full-thickness burn injury, HMGB1 mRNA levels are up-regulated in rat liver and lung beginning 24 h after thermal injury and remain elevated for up to 72 h after a burn [26].

Administration of recombinant HMGB1 in doses that can be found in response to lethal infection mediates lethality in mice. HMGB1 is also toxic to LPS-resistant C3H/HeJ mice, which indicates that HMGB1 mediates lethality even in the absence of endotoxin signal transduction [11]. Doses as low as 250 μg/mouse are sufficient to induce death in both LPS-sensitive and LPS-resistant mice, and doses as low as 1 μg/mouse are sufficient to induce the release of other proinflammatory mediators (TNF-α, IL-1β, and MIP-2) [6]. Histologic examination of HMGB1-exposed lungs reveals acute inflammatory injury, including neutrophil accumulation, interstitial edema, and protein accumulation in the alveolar space [11]. Full-length HMGB1 and the cytokine domain/DNA binding domain B box induce derangements in intestinal barrier function in mice, leading to increased mucosal permeability and bacterial translocation to mesenteric lymph nodes [23]. Intracerebroventricular administration of HMGB1 induces TNF-α and IL-6 production in the brain and mediates taste aversion and anorexia [27].

**HMGB1 inhibitors.** Passive immunization of endotoxin-treated mice with anti-HMGB1 antibodies protects against lethal endotoxemia, even when administered after the peak activity of circulating TNF [6]. Delayed treatment with anti-HMGB1 also prevents the development of lung pathology [11] and attenuates endotoxin-induced anorexia [27]. The protection conferred by anti-HMGB1 does not significantly alter LPS-induced pulmonary levels of TNF-α, IL-1β, and MIP-2, indicating that HMGB1 is an independent mediator of endotoxin-induced inflammation [11]. Anti-HMGB1 antibody administration also rescues mice from lethal sepsis, even when antibodies are administered up to 24 h after cecal puncture. There is no significant HMGB1 release in the serum of these septic mice for ≥12 h, and the antibody is ineffective if dosing is initiated after 36 h (authors’ unpublished data). These parameters define a therapeutic window from the onset of sepsis until ≥24 h later, which is significantly wider than other previously described mediators of sepsis lethality (e.g., TNF [3] and MIF [28]).

Bacterial counts in the spleens of septic animals were not significantly decreased by anti-HMGB1 antibodies, which indicates that the survival advantage conferred was specific and could not be attributed to suppressing bacterial proliferation (authors’ unpublished data). Recombinant bactericidal/permeability-inducing protein (rBPI21) in thermally injured rats attenuated HMGB1 mRNA up-regulation in response to thermal injury, possibly by reducing bacterial exposure [26].

Recently, we identified the “HMGB1 A box,” another DNA-binding domain in HMGB1, as a specific antagonist of HMGB1. Highly purified, recombinant A box (HMGB1 aa residues 1–85) protected mice from lethal sepsis, even when initial A box treatment was delayed for 24 h after disease onset. Signs of sepsis progressed in vehicle-treated controls until death, but A box treatment was associated with recovery and resumption of normal grooming and feeding behaviors. A box competitively displaces the saturable binding of HMGB1 to macrophages (authors’ unpublished data), suggesting that the A box may act as a weak agonist or competitive inhibitor of HMGB1.

Ethyl pyruvate, a relatively nontoxic food additive that attenuates ischemia/reperfusion-induced systemic inflammation [29, 30] also inhibits HMGB1 release. Ethyl pyruvate rescued mice from lethal sepsis, even when dosing was begun as late as 24 h after cecal ligation [31]. Ethyl pyruvate inhibition of HMGB1 is mediated by inhibiting phosphorylation of ERK1/2 and preventing macrophage activation [31]. Thus, 3 independent strategies to inhibit HMGB1 release and action (anti-HMGB1 antibodies, A box, and ethyl pyruvate) each protect against sepsis lethality, even when treatment is initiated as late as 24 h after the onset of sepsis.

**OTHER PHYSIOLOGIC ROLES OF HMGB1**

A growth factor role for HMGB1 was identified by studies of neurite outgrowth. HMGB1 was isolated and sequenced as a developmentally regulated, membrane-associated protein and termed “amphoterin” [32, 33]. Membrane HMGB1 can participate in the regeneration of peripheral neurons by binding to the cell-surface proteoglycan, syndecan, and may play a functional role in the interaction between neurons and Schwann cells [34, 35].

The primary sequences of rodent HMGB1 and amphoterin are identical; rodent HMGB1 is 98% identical to human HMGB1 [8]. HMGB1 is released from cultured murine erythroblasts and is cleaved by a serine protease, producing a 10-aa fragment (aa 129–138) that retains the cell differentiation-stimulating activity of full-length HMGB1. However, this fragment does not express other properties of HMGB1, such
as protein kinase C stimulation or systemic toxicity [24]. On the cell surface, HMGB1 interacts with tissue plasminogen activator (tPA), where it accelerates tPA-catalyzed plasminogen activation. This activity has been implicated in cellular differentiation, regeneration, and migration [7–9]. The HMGB1/tPA interaction is of particular interest in light of the recent introduction of coagulation inhibitors for the clinical management of sepsis. It will be important to determine the effects of anti-HMGB1 antibodies, A box, and ethyl pyruvate on coagulation pathways during sepsis, because diffuse thrombosis and intravascular coagulation is a common final pathway to tissue injury and death from sepsis [1].

### Potential HMGB1 Receptors

HMGB1 binds to macrophages with saturable, specific first-order kinetics (authors’ unpublished data). Membrane-associated HMGB1 can bind to the receptor for advanced glycation end products (RAGE) [36], a member of the immunoglobulin superfamily that was identified in endothelial cells [37]. RAGE is expressed in other tissues, including vascular smooth muscle cells, neurons, and monocytes/macrophages [38]. HMGB1 binds to RAGE with 7 times higher affinity than that of previously known RAGE ligands, advanced glycation end products (AGEs) [37]. RAGE-ligand binding with HMGB1 or AGEs activates nuclear translocation of NF-κB and mitogen-activated protein kinase pathways and can induce migration of rat smooth muscle cells [39] as well as neurite outgrowth [40]. HMGB1-mediated chemotaxis is partially inhibited with anti-RAGE antibodies, implicating RAGE as a receptor that mediates HMGB1-dependent migratory responses [20]. The interaction between HMGB1 and RAGE may also be important in tumor formation, because blocking this interaction can decrease the growth and metastasis of both implanted and spontaneously occurring tumors [41]. A recent study suggests that there may be additional cell surface receptor(s) with which HMGB1 interacts [42].

### Clinical Measurements of HMGB1

HMGB1 may serve as an important marker of critical illness, even in the absence of infection, because its delayed kinetics and large area under the curve may provide an integrated measure of upstream cytokine responses and cell injury. Elevated levels of HMGB1 are observed in humans with sepsis [6] or hemorrhagic shock [43]. Levels are higher in critically ill septic patients than in healthy control subjects and higher in patients who succumb to the disease than in survivors [6]. In nonseptic hemorrhagic shock, serum HMGB1 levels increase within 24 h after the onset of shock and remain high for 72 h; decreasing circulating HMGB1 levels correlate with patient recovery by 96 h [43]. Large prospective studies of serially obtained serum samples in patients with critical illness are ongoing and should reveal whether the kinetics of HMGB1 release are comparable to the delayed pattern in septic and endotoxemic animals and whether serum HMGB1 levels are a marker for previous exposure to early inflammatory mediators.

It appears that HMGB1 released from either injured tissues or activated immune cells may be an immunogen in autoimmune disease. Anti-HMG antibodies have been discovered in the serum of patients with pulmonary hypertension [44], juvenile rheumatoid arthritis [45], drug-induced autoimmunity [46], systemic lupus erythematosus [47], and other autoimmune diseases. HMGB1 is also abundantly expressed in synovial tissues of patients with rheumatoid arthritis, which suggests that HMGB1 may be an important therapeutic target in this disease [48].

### Future Directions

The release of HMGB1 by activated macrophages, its activity as a macrophage-stimulating agent and chemoattractant, and its causative role in lethal endotoxemia and sepsis reveal that HMGB1 is a late cytokine mediator of systemic inflammation. Peak HMGB1 serum activity is detected in a prolonged plateau beginning 12–18 h after disease onset; in contrast, circulating
TNF and IL-1 levels typically revert to near-baseline levels within the first few hours of disease progression. Of importance, specific inhibitors of HMGB1 activity or release (antibodies, ethyl pyruvate, and A box) can rescue animals from lethal systemic inflammation, even when administered as late as 24 h after cecal perforation. These observations suggest that the activity of HMGB1 occurs downstream of TNF and other mediators and may confer a wider therapeutic window in which anti-HMGB1 therapeutics may be effective. Additional studies are required to determine whether this wider window can be translated to therapeutic advantage for human sepsis or other acute inflammatory diseases (table 1).

References


