

EDITORIAL

High Mobility Group Box Chromosomal Protein 1 as a Nuclear Protein, Cytokine, and Potential Therapeutic Target in Arthritis

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High mobility group box chromosomal protein 1 (HMGB-1), a DNA binding protein, is an intracellular protein that facilitates DNA bending, stabilizes nucleosome formation, and modulates the interactions of regulatory molecules with their targets. Recent evidence has revealed that HMGB-1 is also a cytokine, because it 1) is released from activated monocyte/macrophages, 2) mediates delayed endotoxin lethality, 3) activates macrophages, 4) stimulates inflammation *in vivo*, and 5) is a therapeutic target in diverse animal models of inflammatory disease (1–4). Our review accompanies the report by Taniguchi and colleagues (5), in this issue of *Arthritis & Rheumatism*, of a new study of HMGB-1 in the pathogenesis of arthritis. We summarize here the history of research on HMGB-1 as an intracellular protein and discuss in more detail a rapidly growing body of evidence that implicates HMGB-1 as a cytokine that can mediate inflammation and arthritis. Some important new findings are highlighted, and a series of observations that warrant additional study are raised. The molecular basis of the cytokine activity of HMGB-1 and the biochemistry of this potentially important therapeutic target are discussed.

HMGB-1 as a nuclear DNA binding protein and membrane-associated growth factor

HMGB-1, also known previously as high mobility group 1 or amphoterin, was discovered more than 30 years ago as a nuclear chromatin-associated protein and was named originally for its mobility characteristics in polyacrylamide gel electrophoresis (6,7). HMGB-1 is a

single-chain polypeptide with predicted molecular mass of 27 kd. It is a highly conserved, ubiquitous nuclear protein found in eukaryotic cells. The subcellular distribution is tissue-specific: HMGB-1 is located in both nuclei and cytoplasm of undifferentiated tissues, such as lymphoid tissues, testis, neurons, and hepatocytes. Members of the HMGB protein family contain at least one HMG box, a DNA binding domain that defines a family of HMG box-motif nuclear proteins. The HMGB protein family includes HMGB-1, HMGB-2, HMGB-3, and SP100HMG (8,9). HMGB-1 has two such DNA binding domains that enable it to function as a critical cofactor for essential nuclear functions, including DNA bending, transcription, replication, and V-[D]-J recombination. HMGB-1 modulates the activities of nuclear targets, including p53, steroid hormone receptors, glucocorticoid receptor, and homeobox proteins. HMGB-1 is essential for *ex utero* growth and survival; HMGB-1 knockout mice fail to survive more than 24–48 hours postpartum (10).

A growth factor role for HMGB-1 was revealed from studies of a membrane-bound activity expressed in the developing brain and in growing neurites (11,12). Membrane-associated HMGB-1 mediates cellular proliferation and growth by signaling through the receptor for advanced glycation end products (RAGE) (13). Interaction of HMGB-1 with RAGE activates intracellular signal transduction through mechanisms involving nuclear factor κ B (NF- κ B), mitogen-activated protein (MAP) kinases, plasminogen activation, Cdc42, and Rac (11,13).

HMGB-1 as a proinflammatory cytokine

Surprisingly, a cytokine role for HMGB-1 was identified during studies addressing the pathogenesis of endotoxin-induced systemic inflammation (4). The search for a downstream or “late” mediator of delayed endotoxin lethality led to the discovery that HMGB-1 is released, after a significant delay, from macrophage

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cultures activated by exposure to endotoxin, tumor necrosis factor (TNF α), or other proinflammatory stimuli (4). HMGB-1 lacks a classic signal sequence, but large amounts of HMGB-1 are released into the extracellular milieu by activated monocyte/macrophages (14). The mechanism of HMGB-1 secretion is largely unknown, but recent evidence suggests that HMGB-1 can be released via cytoplasmic organelles that exhibit features of endolysosomes (15). Following activation with inflammatory stimuli, monocytes can release large amounts of HMGB-1, but only after a lag or delay of 12–16 hours.

The majority (80%) of the HMGB-1 released by macrophages within the first 16 hours is derived from a preformed cellular pool; after that time, increased cellular synthesis of HMGB-1 leads to incorporation of radiolabeled amino acids into the released protein pool (4). HMGB-1 levels in the serum of endotoxemic animals exhibit kinetics that are delayed significantly compared with those of the classic “early” mediators of endotoxin lethality (TNF α and interleukin-1 β [IL-1 β]). HMGB-1 levels begin to increase ~12–16 hours after the onset of endotoxemia in vivo and remain at an elevated plateau for as long as 48 hours. Very high amounts of HMGB-1 (100–200 ng/ml) have been observed in the serum of animals with endotoxemia (4) and sepsis (16).

Direct evidence for the proinflammatory role of HMGB-1 was obtained by administering highly purified, recombinant HMGB-1 to normal animals and observing the development of inflammation and toxicity. HMGB-1 is lethal to mice, and the toxicity of HMGB-1 synergistically increases the lethality of endotoxin (4). Furthermore, direct administration of recombinant HMGB-1 into the trachea of mice induces an inflammatory response in the lung characterized by neutrophil sequestration, fibrin deposition, and vascular leakage (17). Administration of HMGB-1 to mice mediates the development of fever, anorexia, and “sickness behavior,” a constellation of pathologic responses that are similar to the response to TNF α or IL-1 β (18).

A central role for endogenous HMGB-1 as a proinflammatory mediator was revealed in studies using anti-HMGB-1 antibodies to prevent lethality from endotoxemia (4). Anti-HMGB-1 antibodies conferred significant protection against lethality even when the first dose of antibodies was administered *after* the early TNF α and IL-1 β responses had resolved. Anti-HMGB-1 antibodies have proved to be beneficial when therapy is initiated prior to the onset of increased HMGB-1 levels, a treatment window of 12–16 hours.

Antibodies against HMGB-1 conferred protection against the development of endotoxin-induced acute lung injury, indicating that HMGB-1 is an endogenous mediator of endotoxin lethality and acute lung injury (17).

Recent evidence now indicates that an experimental therapeutic agent, ethyl pyruvate, which attenuates serum HMGB-1 levels, is significantly protective against the lethality of sepsis (19). Ethyl pyruvate was administered to mice with established sepsis caused by cecal ligation and puncture, a standardized and widely used preclinical model of sepsis (19). Administration of ethyl pyruvate significantly reduced serum HMGB-1 levels and conferred a significant survival advantage to mice with established sepsis. Addition of ethyl pyruvate to macrophage cultures significantly inhibited HMGB-1 release following stimulation with endotoxin. Ethyl pyruvate conferred significant protection against lethality even when the first dose was administered 24 hours after the onset of cecal perforation (19). Thus, in vivo studies using either anti-HMGB-1 antibodies or ethyl pyruvate suggest an important role for HMGB-1 as a therapeutic target in acute systemic inflammatory syndromes.

Proinflammatory activities of HMGB-1

Recent observations indicate that HMGB-1 has proinflammatory cytokine activity in vitro. Addition of highly purified recombinant HMGB-1 to macrophage cultures activates the release of TNF α , IL-1, and other proinflammatory mediators (1). Indeed, on a mass basis, HMGB-1 is the most potent endogenous protein yet described as an activator of human and murine macrophages. The kinetics of macrophage activation induced by HMGB-1 are delayed relative to those of classic macrophage activating factors (e.g., TNF α or lipopolysaccharide [LPS]), because addition of HMGB-1 to macrophage cultures leads to increased TNF α release after a significant delay (1). Peak levels of TNF α messenger RNA (mRNA) are not achieved for ~8–10 hours after exposure of monocyte/macrophages to HMGB-1. This time course is significantly delayed compared with the effects of LPS or TNF α ; TNF α mRNA levels are significantly increased within minutes after exposure to stimulating agents (1). Thus, HMGB-1 is characterized both by its delayed action on inducing macrophages to release TNF α and by its own delayed release from activated monocyte/macrophages.

Recent structure–function activity studies of HMGB-1 reveal that the A and B boxes have unique properties (16,20). The cytokine-stimulating domain of HMGB-1 is localized to the B box, because recombinant,

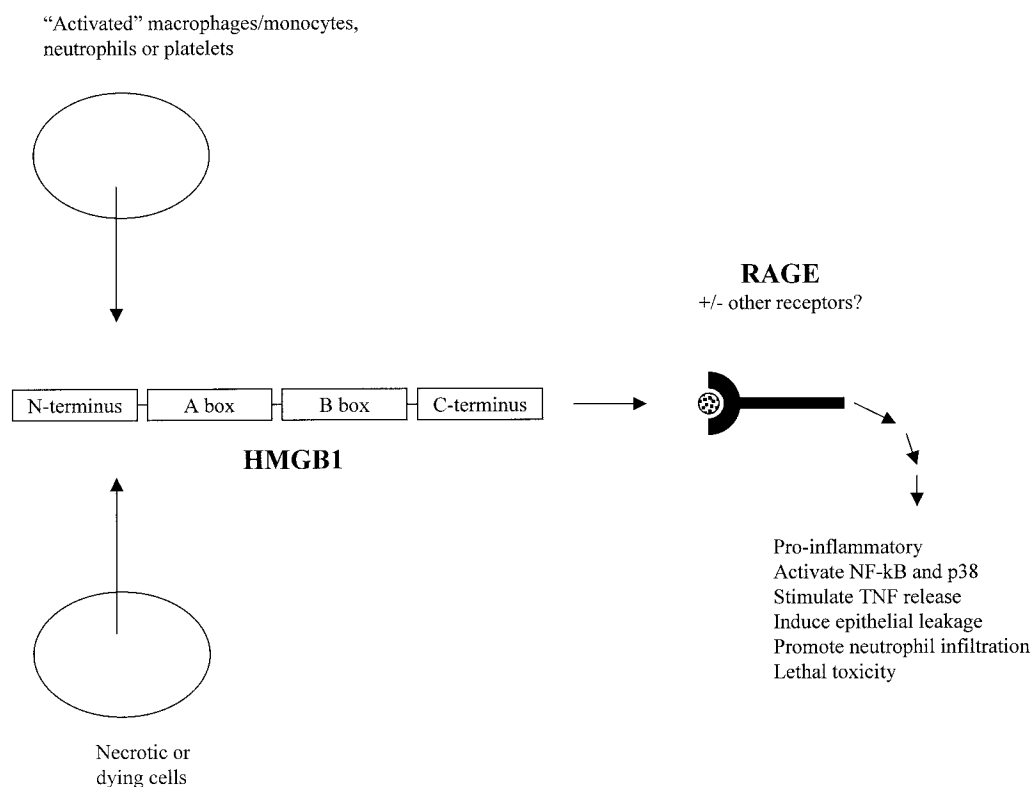


Figure 1. Schematic summarizing some of the current concepts of the structure, function, and activity of high mobility group box chromosomal protein 1 (HMGB-1). RAGE = receptor for advanced glycation end products; NF- κ B = nuclear factor κ B; TNF = tumor necrosis factor.

highly purified B box recapitulates the proinflammatory and macrophage-stimulating activities of full-length HMGB-1 (20). In contrast, purified recombinant HMGB-1 A box antagonizes the proinflammatory activity of full-length HMGB-1 and of the B box itself (16). The A box can function as a competitive antagonist of HMGB-1 binding to target cells (e.g., macrophages), and it remains possible that the A box may also interact with an as-yet-unidentified antiinflammatory receptor. Little is known about whether endogenous biologic processing of full-length HMGB-1 leads to the release of isolated A boxes or B boxes. Figure 1 summarizes some of the current concepts of HMGB-1 structure, function, and activity in inflammatory disease.

HMGB-1 is a primary mediator of inflammation in the setting of cell injury or necrosis. It is widely known that products of injured or damaged cells (as occurs in trauma, cell death, or ischemic injury) activate TNF α release from macrophages and other cells of the innate immune system, but the identity of the stimulating factor(s) released from the injured cells has until now

been enigmatic. Recent evidence using cells devoid of HMGB-1 by gene knockout reveals that deletion of HMGB-1 renders cell lysates significantly less active in stimulating TNF α secretion from monocyte/macrophages (21). Moreover, cells activated to undergo apoptosis release significantly less HMGB-1 compared with necrotic cells, and the diminished HMGB-1 release corresponds to decreased TNF α -inducing activity. Histone deacetylation in apoptotic cells appears to be associated with structural changes in the interaction between DNA and HMGB-1, resulting in decreased HMGB-1 mobility and release (21).

When the data implicating HMGB-1 as a systemic mediator of inflammation are considered, it appears that HMGB-1 is a primary mediator of the acute inflammatory response to cell injury. It is plausible that HMGB-1 may be an "integrator" of systemic inflammatory responses, because cell injury from a variety of sources can contribute to systemic increases in HMGB-1. For example, HMGB-1 levels derived from activated monocytes, from ischemic or injured cells, or

from necrosis can cause a systemic inflammatory response in clinically diverse states, including endotoxemia, sepsis, and multiple organ injury from trauma or infection (2,3).

An important pathobiologic activity of HMGB-1 was recently revealed in studies performed by Sappington and colleagues, which implicated HMGB-1 as a mediator of epithelial barrier dysfunction (22). Exposure of epithelial cell monolayers to HMGB-1 caused the monolayers to become "leaky," so that large, normally excluded molecules passed through the cell layer. Epithelial leakage mediated by HMGB-1 was not attributable to toxicity, but required signaling through MAP kinases, NF- κ B, and nitric oxide (22). This finding reveals a potentially important mechanism of HMGB-1 toxicity. Histopathologic study of animals that have died of acute HMGB-1 poisoning reveals surprisingly sparse changes and minimal evidence of necrotic injury, acute inflammation, or necrosis (4). It may be that epithelial dysfunction, occurring systemically in response to excessive HMGB-1 levels, underlies the development of lethality when serum HMGB-1 levels are elevated.

HMGB-1 levels in disease

Serum HMGB-1 levels are <5 ng/ml in the serum of healthy animals and normal human subjects when assayed using a quantitative immunoblot based on neutralizing anti-HMGB-1 antibodies and a standard curve constructed with recombinant HMGB-1. Very high circulating levels of HMGB-1 (up to 150 ng/ml) are observed in patients with severe sepsis, and the highest levels were observed in patients who died (4). Hemorrhagic shock is also associated with significantly increased serum levels of HMGB-1, even in the absence of infection or endotoxemia (23). This finding indicates that the stimulus to HMGB-1 release may be either proinflammatory cytokines released by ischemia or ischemia-induced cell injury.

HMGB-1 is subject to extensive posttranslational modification, including glycosylation, phosphorylation, and acetylation, but native HMGB-1 has not been isolated from pathologic material (e.g., serum or synovial fluid), and it is not known whether the "pathologic" protein differs significantly from HMGB-1 found in normal conditions. Native HMGB-1 purified from macrophages is active as a proinflammatory stimulus to macrophages, suggesting that, even without modification, HMGB-1 can function as an inflammatory agent.

HMGB-1 and arthritis

Recent evidence implicates HMGB-1 in the pathogenesis of arthritis. Immunohistochemical staining for HMGB-1 performed on synovial tissue obtained from mice and rats with collagen- or adjuvant-induced arthritis reveals that HMGB-1 expression is significantly increased extracellularly and in the cytoplasm of macrophage-like cells (24). This pattern differs significantly from the strictly nuclear HMGB-1 staining pattern observed in synovial cells obtained from normal mice and rats. Moreover, synovial fluid HMGB-1 levels are significantly elevated in patients with rheumatoid arthritis, indicating increased extracellular levels of this putative proinflammatory mediator in human arthritic joints (24).

In this issue of *Arthritis & Rheumatism*, Taniguchi and coworkers (5) confirm significantly increased levels of HMGB-1 in the synovial fluid of rheumatoid arthritis patients. HMGB-1 levels were significantly higher in rheumatoid arthritis synovial fluid than in osteoarthritis synovial fluid. Synovial fluid HMGB-1 levels in rheumatoid arthritis patients were ~ 50 ng/ml, while those of osteoarthritis patients were ~ 10 ng/ml. Synovial fluid macrophages exhibited increased expression of RAGE and could be activated to release TNF α , IL-1 β , and IL-6 by exposure to HMGB-1. Exposure of CD68-positive cells from the rheumatoid arthritis synovium to TNF α activated a translocation of HMGB-1 from the nucleus to the cytosol.

In accordance with findings of Kokkola and colleagues (24), Taniguchi et al report that HMGB-1 is localized primarily in the cytosol of macrophage-like cells located in the sublining layer. The cells exhibited the characteristics and expected distribution of synovial macrophages, confirmed by the fact that cells with cytosolic HMGB-1 expression coexpressed CD68 surface molecules. HMGB-1 release from synovial fluid macrophages was enhanced by exposure of the cultures to TNF α . Thus, synovial monocyte/macrophages in arthritic joints can respond to inflammatory agents to release HMGB-1 and can be activated by HMGB-1 to release proinflammatory cytokines.

Taniguchi and colleagues were unable to detect increased serum levels of HMGB-1. As noted above, the specific methodology and antibody reagents can have an important impact on the results of these types of measurements. We recently measured plasma HMGB-1 concentrations in 44 patients with active rheumatoid arthritis by quantitative immunoblot using a neutralizing anti-HMGB-1 antibody. Plasma samples were size-

fractionated by ultrafiltration through a 100-kd membrane to remove high molecular weight proteins prior to analysis. In contrast to the results of the Japanese study (5), we observed increased HMGB-1 levels in plasma samples from rheumatoid arthritis patients (mean \pm SD 80 ± 11 ng/ml, range 7–240) (24). In this small study, we did not observe a correlation with standard measures of disease activity, such as tender and swollen joint counts, raising the possibility that the observation of increased HMGB-1 levels using our methodology may be identifying a pathophysiologic subset of rheumatoid arthritis patients rather than simply reflecting the level of disease activity. This finding would be consistent with the genetic heterogeneity that appears to underlie rheumatoid arthritis and other autoimmune inflammatory disorders. Significantly, however, HMGB-1 levels are increased in the circulation during human rheumatoid arthritis.

Considered together, these data indicate that HMGB-1 can contribute to the pathogenesis of chronic inflammatory diseases, including arthritis. HMGB-1 stimulates synovial macrophages to produce and release TNF α , IL-1 β , and IL-6; it can bind to components of the plasminogen activation system and enhance the activity of tissue plasminogen activator and of matrix metalloproteinases (MMPs), such as MMP-2 and MMP-9 (25). It is plausible that HMGB-1 plays a role in both the inflammatory and destructive processes in the pathophysiology of arthritis. Prominent features of chronic synovitis are necrotic cell death and activation of macrophages. Both of these processes release HMGB-1, which can contribute to the inflammation of chronic arthritis. Future studies will be needed to determine the primary and secondary events of this role of HMGB-1 in disease pathogenesis.

Future HMGB-1 research in arthritis

These findings raise several important questions about the role of HMGB-1 in arthritis. One important aspect is whether the circulating form of HMGB-1 implicated in the pathogenesis of inflammation exhibits unique biochemical characteristics or modifications compared with the material found normally in healthy cells. The cell source(s) of circulating HMGB-1 is not known, but probably includes monocyte/macrophages or platelets that have been activated by inflammatory stimuli, and injured or dying cells that release HMGB-1 during cell lysis (4,21,26). It is not yet known whether modification to HMGB-1 during processing or release is important to its proinflammatory, cytokine activity. Notably, HMGB-1 is a charged, carrier molecule which may

be bound to other biologically active factors in the extracellular milieu. HMGB-1 efficiently binds DNA, and anti-HMGB-1 antibodies have been detected in the plasma of patients with autoimmune disease (27–29). As with other cytokines, circulating factors or binding antagonists may modulate the activity of HMGB-1. These putative factors could also influence the ability to detect HMGB-1 in clinical samples, since these assays depend on the methods of sample preparation and fractionation and on the activity of antibodies.

Another major consideration raised by these studies is the relationship between the proinflammatory activity of HMGB-1 and that of TNF α , a validated therapeutic target for rheumatoid arthritis. It is not clear whether HMGB-1 signaling requires the release of TNF α or whether TNF α signaling depends on the release of HMGB-1. Indeed, in some pathologic scenarios, these interacting cytokines may require each other to manifest full cytokine activity. Alternatively, these proinflammatory cytokines may act alone, each independently of the other; if this proves to be the case, TNF α and HMGB-1 responses may prove useful for defining a new method to stratify or characterize clinical subtypes of arthritis. Therapeutic intervention with neutralizing anti-HMGB-1 antibodies in experimental arthritis models may help to clarify this issue. It will also be important to delineate whether plasma and synovial fluid HMGB-1 levels in arthritis patients are influenced by TNF α -blocking therapy.

Finally, additional research is needed to fully understand HMGB-1 signaling during inflammation. RAGE has been implicated as an HMGB-1 receptor, but it is unclear, for instance, how signaling through RAGE, which activates intracellular signal transduction pathways within minutes, can lead to a delayed expression of TNF α mRNA occurring several hours later. A recent study of structural and functional RAGE polymorphisms does not support a major role for this receptor in disease susceptibility (30). The possibility has been raised that other receptors may participate in signaling the inflammatory effects of HMGB-1. Future studies on the mechanism of HMGB-1, and of the potential to therapeutically inhibit the activity or release of HMGB-1, may lead to the development of new diagnostic and therapeutic agents for chronic inflammatory diseases, such as arthritis.

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