Abstract

Since its identification a third of a century ago, the high-mobility group box-1 (HMGB1) protein has been linked to varied diverse cellular processes, including release from necrotic cells and secretion by activated macrophages engulfing apoptotic cells. Initially described as solely chromatin-associated, HMGB1 was additionally discovered in the cytoplasm of several types of cultured mammalian cells 6 years later. In addition to its intracellular role, HMGB1 has been identified extracellularly as a putative leaderless cytokine and differentiation factor. In the years since its discovery, HMGB1 has also been implicated in disease states, including Alzheimer’s, sepsis, ischemia-reperfusion, arthritis, and cancer. In cancer, overexpression of HMGB1, particularly in conjunction with its receptor for advanced glycation end products, has been associated with the proliferation and metastasis of many tumor types, including breast, colon, melanoma, and others. This review focuses on current knowledge and speculation on the role of HMGB1 in the development of cancer, metastasis, and potential targets for therapy.

The closing years of life are like the end of a masquerade party, when the masks are dropped.

Cesare Pavesi

The high-mobility group box-1 (HMGB1) protein is present in almost all eukaryotic cells. It was first identified by Goodwin, Sanders, and Johns in 1973 as one of a “group of chromatin-associated protein with high acidic and basic amino acid contents” (1). As an ancient protein that probably originated before the split between the animal and plant kingdoms more than 500 million years ago (2), it is ubiquitously present in the nucleus of almost all mammalian cells and is highly conserved between species (3). In 1979, Michael Bustin showed that HMGB1 could also be found in the cytoplasm (4, 5). Because then, we have come to recognize many additional functions possessed by this versatile protein. Within the nucleus, HMGB1 stabilizes nucleosomes and regulates transcription of many genes (6, 7). As a cytokine-like factor, HMGB1 is secreted by macrophages (8, 9), mature dendritic cells (ref. 10), and natural killer cells (11) in response to injury, infection, or other inflammatory stimuli. The biological importance of HMGB1 is underscored by its multifunctionality, as well as pathologic conditions in man implicated by its dysregulation: Alzheimer’s Disease (12), sepsis (13), ischemia-reperfusion injury (14), arthritis (15), and cancer (16). Here we will focus on current knowledge concerning the role of HMGB-1 in the development of cancer, metastasis, and as a potential target for therapy.

HMGB1 Gene/Protein and Regulation of Expression

The HMGB1 gene consists of six exons and is located on the human chromosome 13q12 (17). Southern blot analysis of the human genomic DNA reveals several pseudogenes and one active gene within the genome (18). Expression of the human HMGB1 gene is under the control of a very strong TATA-less promoter, which has one major start site at 57 nucleotides upstream from the first exon-intron boundary. This TATA-less promoter is one of the highest expressing mammalian promoters and exhibits an 18-fold greater transcriptional activity than that of the SV40 promoter. Immediately, upstream of the transcriptional start site is a silencer element that represses the transcriptional activity of the HMGB1 promoter by >80%. The transcriptional repression of the silencer element is offset by an enhancer element found in the first intron of the human HMGB1 gene and can increase the HMGB1 activity by 2-fold to 3-fold (19).

Members of the HMG superfamily are typically 25 to 30 kDa with homologous basic domains that mediate DNA binding, the HMG boxes (20). The human HMGB1 gene encodes a 215-amino acid polypeptide. HMGB1 is highly conserved among species, with over 98% identity between rodent, bovine, and human proteins (21, 22). Structurally, HMGB1 is composed of three domains: two positively charged domains (A and B boxes) and a negatively charged carboxyl terminus (acidic tail; Fig. 1; refs. 23, 24). Functionally, A and B boxes are DNA-binding domains. The B box contains the cytokine-like activity, inducing macrophage secretion of additional proinflamatory cytokines (25). This cytokine activity is antagonized by recombinant A box. The first 21 amino acid residues (26–46) of the recombinant B box represent the minimal peptide sequence that retains the cytokine-like activity. The protein structure involved in the binding of HMGB1 with...
receptor for advanced glycation end products (RAGE) is located between amino acid residues 150 and 183.

HMGB1 expression and subcellular localization varies in individual cell types and during cell activation (47). Control of HMGB1 gene transcription has been linked to several steroid hormones involved in development, including glucocorticoids, estrogens, and progestins. HMGB1 transcription is up-regulated by IFN-γ, tumor necrosis factor (TNF)-α, and transforming growth factor-β in matured THP-1 macrophages and in human peripheral blood monocytes. Posttranscriptional control of mRNA stability or translation is likely important as well (47).

HMGB1 undergoes posttranslational modifications, such as phosphorylation and ADP ribosylation; whereas acetylation (48, 49) had potential modifications. ADP ribosylation of HMG proteins regulates gene transcription and may be necessary for cell recovery from DNA damage (50, 51). Acetylation allows for the generation of tetramer formation of histone-histone complexes and the stimulation of complex formation with DNA polymerase α in the process of DNA replication (52). Acetylation by histone acetyltransferases at the K2 position results in enhanced affinity to distorted DNA (49, 53). DNA bending ability is attributed to modification of K81 located in close proximity to B box (49, 54, 55). Acetylation modulates HMGB1 interactions with other proteins, regulates entry into the nucleus, determines several biological activities, and regulates its inflammatory potential (56).

HMGB1 as a Nuclear Protein

HMGB1 binds the minor groove of DNA facilitating the assembly of site-specific DNA-binding proteins, including nuclear hormone/nuclear hormone receptor complexes and p53 or p73 transcriptional complexes (57, 58). As it binds DNA, nuclear HMGB1 plays an important role in the facilitation of protein-protein interaction and recognition of DNA damage in the process of mismatch repair (59). HMGB1 acts to recruit proteins required for the early steps in mismatch repair enabling physical interactions with exonucleases, helicases, polymerases, and ligases at or before the excision of mispaired nucleotides (59). HMGB1 enhances heteroduplex bending by exonucleases, exposing the DNA substrate to attack by helicases and/or nucleases (59).

Nuclear HMGB1 also facilitates the DNA-binding and transcriptional activity of steroid receptor members of the nuclear receptor family of transcription factors, in particular, the estrogen receptors ERα and ERβ. Both are responsive to HMGB1 via enhancement of receptor DNA-binding affinity and transcriptional activity dependent on the C-terminal extension region of the estrogen receptor DNA-binding domain (60). In its role as a “superadaptor,” HMGB1 has been proposed to act through “hit-and-run” mechanism with these steroid hormone receptors. It has been suggested that the HMGB protein acts as a transient and unstable component of
the estrogen receptor/estrogen response element complex, enhancing binding interactions at two interfaces. Through its binding in the vicinity of the estrogen response element to open the minor groove, HMGB1 allows for rearrangement of the C-terminal extension residues. Overall this facilitates access to and interactions in the minor groove, which is energetically less accessible in the absence of HMGB1. Due to the fact that the estrogen receptor and HMGB1 proteins are undergoing a dynamic association and dissociation with DNA, the estrogen receptor maintains this binding affinity only as long as HMGB1 remains available. Through this mechanism, these data suggest that HMGB1 proteins may play a critical role in the enhancement of the binding of steroid hormone receptors to their cognate response elements and the regulation of many estrogen-responsive genes and a vital role in the biology of estrogen-sensitive cancers (61).

In the context of cancer, nuclear HMGB1 is responsible in part for the efficacy of platinum-based therapies through its affinity for DNA adducts, preventing interaction with the mutation repair machinery. In particular, HMG domain proteins inhibit repair of the 1,2 intrastrand d(GpG) crosslink by the human excision nuclease, suggesting that levels of HMG proteins in tumors may influence susceptibility to platinum therapy and serve as a potential screen for therapeutic efficacy (62). However, the effect of HMGB1 on platinumated DNA remains unclear, as cisplatin-resistant human cancer cells mediate resistance through enhanced expression of the HMGB1 gene product (63).

HMGB1 as a Masquerader: Taking on an Extracellular Role

HMGB1 release into the extracellular environment is mediated by active and passive mechanisms. It has been suggested that HMGB1 is hyperacetylated on many of its 43 lysine residues to allow migration into the cytosol (16), although this has yet to be confirmed by others. This enzymatic modification presumably allows HMGB1 to “change its mask” and acquire a role as a cytokine-like agent. From the cytosol, HMGB1 is released into the extracellular environment as a leaderless cytokine. It is synthesized without a leader sequence, which is used by conventional cytokines to allow delivery into the rough endoplasmic reticulum, and Golgi apparatus for release through the cell membrane. Instead, HMGB1 release is possibly mediated by specialized organelles that belong to the endolysosomal compartment (64). Active secretion initiates execution of cellular response programs in activated macrophages and monocytes (56). Via nuclear localization signals 1 and 2, HMGB1 is translocated from the cytosol of cells into the nucleus to bind DNA. During the immunologic challenge, macrophages are activated, resulting in the acetylation of nuclear localization signal sites within HMGB1-enabling cytosolic retention, followed by packaging into secretory lysosomes and liberation into the extracellular environment (Fig. 2; refs. 8, 64).

Passive release of HMGB1 into the extracellular space occurs during unscheduled cell death, where HMGB1 diffuses out of the leaky membranes of the necrotic cells (65). In this role, HMGB1 serves as a “necrotic marker” or damage-associated molecular pattern for recognition by cells of the innate immune system and is a surrogate for the extent of injury initiating tissue repair (56). Passive release of HMGB1 is not observed during type I apoptotic cell death, in which HMGB1 is tightly sequestered within apoptotic bodies, thereby preventing its release. Apoptotic cellular chromatin is not only digested at nucleosomal intervals but also is hypoacetylated with phosphorylation of histone H2B (47), enhancing tight HMGB1 binding.

Outside the cell, HMGB1 masquerades as a cytokine to activate endothelial cells (Fig. 3), promoting angiogenesis and extravascular emigration of inflammatory cells and stem cells, thereby initiating inflammation (66, 67). In this extracellular role, HMGB1 induces concentration-dependent activity as an early mediator after acute injury and late mediator of other varied inflammatory processes (68). Like other cytokines, it has...
both beneficial and pathologic effects, confining infection and damage and allowing healing and regeneration of injured tissues (69). Monocytes, macrophages, natural killer, and myeloid/plasmacytoid dendritic cells secrete HMGB1 in response to pathogen or damage-associated molecules with resultant extracellular HMGB1-recruiting myeloid dendritic cells, plasmacytoid dendritic cells, macrophages, neutrophils, and CD4+ T cells. In its role as a late acting mediator of inflammation, HMGB1 induces the release of proinflammatory cytokines from monocytes (9, 65), such as interleukin (IL)-12, IL-6, IL-1α, and IL-8, and matures myeloid dendritic cells, up-regulating CD40, CD54, CD80, CD83, and MHC class II molecules (70, 71). In endothelia, dose-dependent HMGB1 stimulation results in increased expression of intercellular adhesion molecule 1, vascular cell adhesion molecule, RAGE, tissue-type plasminogen activator, IL-8, TNF (52), MCP-1, and plasminogen activator inhibitor 1 (72). In endothelia, dose-dependent HMGB1 stimulation results in increased expression of intercellular adhesion molecule 1, vascular cell adhesion molecule, RAGE, tissue-type plasminogen activator, IL-8, TNF (52), MCP-1, and plasminogen activator inhibitor 1 (72). In primitive mesangioblasts and bone marrow–derived stem cells, HMGB1 serves as a migration target from damaged tissues, thereby promoting wound repair (71).

Excessive extracellular amounts of HMGB1 can also cause uncontrolled inflammatory responses leading to tissue injury/organ failure, disruption of endothelial barrier functions, vascular leakage, and tissue hypoperfusion (69). Pretreatment with HMGB1 in ischemia-reperfusion models decreases subsequent inflammatory responses, demonstrating the ability of HMGB1 to limit innate immune mechanisms (73).

**HMGB1 as a Regulator of Cell Motility and Metastasis**

In 1991, a membrane-bound molecule expressed on growing neurites was identified by Rauvala et al. and named amphoterin based on its positively charged amino terminal domains and a negatively charged carboxy terminus (74). Membrane-bound amphoterin activates RAGE-expressing cells, leading to neurite outgrowth and survival in part through increased expression of B-cell lymphoma 2. In addition to the role of HMGB1 in neurite outgrowth, additional studies have suggested a role in cell motility and invasive behavior (75). This notion is supported by observations that HMGB1 is up-regulated in immature cells, migrating growth cones, and edges of migrating malignant cells (75, 76). HMGB1 expression is high in migrating cells but is down-regulated as a consequence of cell-to-cell contact as a sensor for contact-dependent inhibition in migrating cells (75, 77). HMGB1 also binds tissue-type plasminogen activator and plasminogen through their lysine-binding kringle domains, resulting in plasmin production and enhancing penetration and invasion into tissues (75, 76, 78).

**HMGB1 as a Superadaptor Promoting Interaction with Receptors and Signaling**

HMGB1 signals alone or together with other molecules as a linker or superadaptor. HMGB1 binds heparin (75), syndecan-1 (79), and phosphacan (80), acting to link phosphacan to cell surface adhesion molecules (80). HMGB1 is a sticky protein and is postulated to exist in a “molten globule-like” state that facilitates its binding to other proteins.

Several receptors have been identified for HMGB1 including RAGE, toll-like receptors (TLR) 2 and 4, syndecan (79), a specific receptor, tyrosine phosphatase, (80), and thrombomodulin (Tables 1 and 2; ref. 81).

HMGB1 binds RAGE expressed on neurons, endothelium, smooth muscle, monocytes, macrophages, T cells, and immature...
dendritic cells (56). As a member of the immunoglobulin superfamily, the RAGE gene, like TNF and lymphotoxin, is located within the MHC class III region near the junction with the class II MHC complex in humans and mice (82, 83). It is composed of three extracellular immunoglobulin-like domains, a single pass transmembrane domain, and a short highly charged cytoplasmic domain essential for signal transmission (84). RAGE expression mirrors developmental processes with high expression during embryonic development and down-regulation with age (85–87). RAGE increases are observed at the extremes of age, possibly due to the accumulation of its ligands or as a compensatory protective mechanism (86, 87). After RAGE interaction, HMGB1 induces phosphorylation of extracellular signal-regulated kinase (ERK; ref. 88) and nuclear translocation of nuclear factor-κB (NF-κB; ref. 56) induces neurite outgrowth (85) and activates GTPases of the Rho family, Cdc42 and Rac, implicated in the cytoskeletal remodeling in cell movement (84). HMGB1 induces a proangiogenic phenotype in endothelial cells after activation of ERK1/ERK2 intracellular signaling via binding of extracellular RAGE and other cell surface receptors (88). When triggered by extracellular HMGB1, RAGE mediates endothelial cell activation (89), smooth muscle cell migration (90), and mesangioblast migration and proliferation (91). Interestingly, RAGE knockout models result in a phenotype of decreased innate immunity but intact adaptive immunity as illustrated by the finding that RAGE deletion provides protection from the lethal effects of septic shock but does not protect from inflammatory reactions such as those in delayed-type hypersensitivity (92). HMGB1 knockout mice display a phenotype of normal organ development and cell growth but die within 24 h of birth from hypoglycemia (93). Cell lines lacking HMGB1 grow normally, but the activation of gene expression by the glucocorticoid receptor (encoded by the gene *Grl1*) is impaired. This suggests that HMGB1 is critical for proper transcriptional control by specific transcription factors (93).

Table 1. Cancer prognosis and correlated HMGB1-RAGE signaling

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Selected references</th>
<th>Comments</th>
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<tbody>
<tr>
<td>Breast</td>
<td>(36, 58)</td>
<td>Increased HMGB1 protein expression occurs in breast carcinomas compared with normal tissue. Varying responses of estrogen positive breast cancer may depend on HMGB1 as a modulator of estrogen receptor/estrogen response element binding.</td>
</tr>
<tr>
<td>Colon</td>
<td>(30–35)</td>
<td>HMGB1 has been defined as an antiapoptotic oncoprotein in human colon cancer due to its over expression and inhibition of apoptosis. Levels are extremely elevated in tumor samples compared with normal tissue, along with the antiapoptotic NF-κB gene product c-IAP2. Coexpression of RAGE and HMGB1 enhanced migration and invasion by colon cancer cell lines. Colon cancer lines expressing HMGB1 have also been a model illustrating that macrophage infiltration into cancers is suppressed, providing a mechanism for tumor destruction of the innate immune system. RAGE expression is associated with atypia, adenoma size, metastasis to lymph nodes/distal organs, and poor prognosis at any stage.</td>
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<tr>
<td>Melanoma</td>
<td>(20, 106-108)</td>
<td>HMGB1 is linked to the initial development of melanoma and is strongly up-regulated in melanoma compared with primary melanocytes. HMGB1 binds to the melanoma inhibitory activity promoter to enhance promoter activity in melanoma cells, with HMGB1 up-regulation closely paralleling malignant transformation.</td>
</tr>
<tr>
<td>Prostate</td>
<td>(27, 63)</td>
<td>Expression of HMGB1 and RAGE post luteinizing hormone – releasing hormone therapy was increased in metastatic cases, however not in nonmetastatic cases. The majority of metastatic cases showed coexpression of RAGE and HMGB1 in tumor cells and stromal cells.</td>
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<tr>
<td>Pancreatic</td>
<td>(27, 63)</td>
<td>Human pancreatic cancer lines with high metastasis ability displayed strong expression of RAGE and MMP-9 compared with normal cell lines. MMP-9 and RAGE are now considered indicators of metastasis ability. HMGB1 has been officially grouped as a metastasis specific gene in pancreatic cancer.</td>
</tr>
<tr>
<td>Lung</td>
<td>(37–40, 103)</td>
<td>Provides an exception to the norm of HMGB1-RAGE over expression in human tumors. RAGE-HMGB1 is down-regulated compared with normal control tissues. sRAGE administration did not suppress tumor growth as typically found in other tumors. RAGE is strongly reduced at both the mRNA and protein levels with down tumors. RAGE is strongly reduced at both them RNA and protein levels with down-regulation paralleling higher tumor stages. Down-regulation was also observed in benign neoplasms.</td>
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NOTE: The role of HMGB1 in cancer varies depending on the tumor type. HMGB1-RAGE interaction is typically up-regulated in tumors and is linked to metastasis and poorer prognosis. Lung tumors, however, show down-regulation of RAGE and HMGB1, and administration of lung tumors with RAGE and HMGB1 causes decreased proliferation and metastasis.
Transfection experiments suggest that TLR2 and TLR4 receptors are also involved in HMGB1-induced NF-κB activation (14, 56). The ability of necrotic cells to maximally stimulate NF-κB activity in macrophages is at least in part dependent on a TLR-mediated signaling pathway; and both TLR2 and TLR4 signaling have been implicated in the activation of macrophages by HMGB1 (14). Signaling through TLRs has also been suggested as a mechanism of HMGB1 induction of neovascularization during periods of innate immune system activation (81, 94). HMGB1, through TLR activation, increases maturation and cytokine secretion from myeloid and plasmacytoid dendritic cells, initiating secretion of IL-18. Natural killer cell secretion of HMGB1 drives dendritic cell maturation, in turn protecting dendritic cell from IL-18. Natural killer–mediated lysis. HMGB1 is secreted by activated macrophages and monocytes after exposure to lipopolysaccharide, TNF-α, or IFN-γ, but not migration inhibitory factor, IL-6, or macrophage inflammatory protein (MIP)-1β (56).

Thrombomodulin-HMGB1 interactions have also been linked to neovascularization (Fig. 4). Thrombomodulin is an endothelial cell membrane glycoprotein (95, 96) containing five domains: D1 (an N-terminal lectin-like region with antiinflammatory properties, mediating cell adhesion and binding HMGB1; ref. 97), D2 (a region consisting of six epidermal growth factor–like structures, critical for antiangiogenic activity), D3 (a glycosylated domain), D4 (a transmembrane region), and D5 (the cytoplasmic tail; ref. 96). The N-terminal domain of thrombomodulin sequesters HMGB1, inhibiting its interactions with RAGE. Increased serum thrombomodulin and impedance of HMGB1 proangiogenic effects through RAGE has been suggested as a cause of poorer outcomes after acute coronary syndrome (97, 98). Recently, extremely elevated levels of thrombomodulin have been identified in the setting of myocardial infarction and stroke (99).

### Role of HMGB1 in Cancer

The notion that necrotic tumor cells could provide signals to enhance the growth of remaining viable ones has been considered for over 50 years (100). The initial discovery of HMGB1-RAGE interactions in the modulation of neurite outgrowth suggested that HMGB1-RAGE signaling might also be involved in cancer metastasis. Indeed, HMGB1-RAGE interactions mediated invasion, migration, and the growth and spread of implanted C6 gliomas (101). Blockade of this interaction by soluble or mutated RAGE resulted in suppression of migration of the glioma, forcing the tumor to undergo prolonged dormancy with decreased proliferation, invasion, and matrix metalloproteinase (MMP) activity (102). Initially it was hypothesized that increased RAGE-HMGB1 activity was elevated in all cancers; however, recent studies have revealed that this increase is not always the case, such as in lung cancer, in which reduced RAGE expression is found (Table 3; ref. 103).

With confirmation of increased HMGB1 levels in most tumor cells, studies were done to determine whether HMGB1 varied in tumor samples when compared with that found in normal cells. HMGB1 from Guerin ascites tumor cells were five times more poly(ADP) ribosylated when compared with control rat liver cell HMGB1 (104). Tumor HMGB1 was also highly acetylated, whereas normal tissue HMGB1 was not. Although acetylation can induce conformational changes and HMGB1 oligomerization, this change was later identified as an attribute

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**Table 2. HMGB-1 Receptors and signaling**

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<thead>
<tr>
<th>Receptor</th>
<th>Comment</th>
<th>Reference</th>
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<tr>
<td>RAGE</td>
<td>Interactions with HMGB1 result in MAP kinase activation, enhanced tumor growth, metastases, and release of MMPs. RAGE-HMGB1 also interact with the Rho family of GTPases, Cdc42 and Rac to regulate cell motility. Activates Ras and NF-κB (members of the MAP kinase serine/threonine phosphorylation pathway) in inflammatory responses.</td>
<td>(84, 102)</td>
</tr>
<tr>
<td>TLR2/TLR4</td>
<td>Interaction with HMGB1 results in activation of macrophages and maximal stimulation of NF-κB activity in macrophages, along with induction of neovascularization in periods of innate immune system activation.</td>
<td>(73, 94)</td>
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<tr>
<td>Syndecan</td>
<td>HMGB1 links syndecan to the ECM, participates in cell adhesion and migration of simple epithelial cells, particularly in early cell spreading.</td>
<td>(79)</td>
</tr>
<tr>
<td>Phosphacan/protein-Tyr phosphatase γ/β</td>
<td>This receptor participates in proteoglycan-mediated regulation of cell adhesion, neurite growth and cell migration during central nervous system development. It links phosphacan in the extracellular matrix or the transmembrane phosphatase on adjacent cells, to cell surface glycoproteins such as contactin to which phosphacan alone binds only minimally.</td>
<td>(80)</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>This activates plasmin via formation of ternary complexes, also binds tissue-type plasminogen activator.</td>
<td>(78)</td>
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**NOTE:** Many studies have illustrated the ability of HMGB1 to signal alone or in concert with other molecules as a linker. Several receptors have been identified for HMGB1 including RAGE, TLR2, TLR4, syndecan (58), a specific receptor-tyrosine phosphatase (59), and thrombomodulin (60). The signaling mechanisms and end events of these receptor interactions are still not totally clear.
of proliferating tissues in general and not restricted to tumor cells (104). Whether HMGB1 can be linked to general cellular proliferation or solely metastasis is still debated. At minimum, HMG proteins are involved in transcriptional regulation of a number of genes with potential roles in malignancy, such as TNF (105).

Melanoma. Changes in transcriptional regulation represent an early event in tumorigenesis arising within the tumor microenvironment after epigenetic and genetic changes in the tumor cell (20). Melanoma development and progression is linked to several transcription factor modifications, including cAMP-responsive element binding protein, C-terminal binding protein, microphthalmia transcription factor, NF-κB, as well as HMGB1 (20). Melanoma cell lines overexpress HMGB1 at the mRNA and protein levels when compared with control melanocytes, and this is linked to the development of melanoma (106). HMGB1 binds to a specific region within the melanoma inhibitory activity promoter to enhance promoter activity in melanoma cells, with repression of HMGB1 expression leading to suppression of cell invasion (20). In melanoma cells, HMGB1 up-regulates expression of the melanoma inhibitory activity protein, which closely parallels malignant transformation (106). Melanoma inhibitory activity expression in vivo correlates with the progressive malignancy of melanocytic tumors (107, 108), further confirmed by finding increased melanoma inhibitory activity protein levels in patients with metastatic melanomas (26, 109).

Pancreatic cancer. Several human pancreatic cancer cell lines with varying metastatic potential were examined for expression of RAGE and MMP-9, both associated with tumor progression and metastasis (27). Lines with high metastatic ability displayed strong expression of RAGE and MMP-9 compared with minimal expression of both proteins in nonmetastatic lines. For this reason, MMP-9 and RAGE are indicators of metastatic ability in pancreatic cancer cells of possible therapeutic importance (27).

Colon cancer. Overexpression of HMGB1 in human colon carcinoma with resultant inhibition of apoptosis suggests the role of HMGB1 as an antiapoptotic oncoprotein (30). HMGB1 protein levels are significantly elevated in human colon carcinomas when compared with control matched normal tissues, leading to increased NF-κB activity and increased expression of the antiapoptotic NF-κB target gene product c-IAP2 (inhibitor of apoptosis), which acts to suppress TNF (30). IAP proteins bind and inhibit caspases resulting in the downstream prevention of cytochrome C release in mitochondria and complete inhibition of the apoptosis pathway (30). Suppression of caspase-9 and caspase-3 activity is linked to HMGB1 overexpression, providing

Prostate cancer. Expression of HMGB1 and RAGE was examined in prostatectomy specimens from 40 patients with pT3 prostate cancer (metastatic and nonmetastatic) preoperatively treated with luteinizing hormone–releasing hormone agonist (28). HMGB1 expression was detected in cells from 27% of metastatic cases and in none of the nonmetastatic cases. HMGB1 was detected in prostatic stromal cells of 63% derived from metastatic cases compared with 11% nonmetastatic cases. RAGE expression was detected in cells from 73% of metastatic cases and 33% of nonmetastatic cases (P = 0.0244). Most metastatic cases coexpressed both HMGB1 and RAGE in tumor cells or in tumor and stromal cells (73%). Androgen deprivation provided by the luteinizing hormone–releasing hormone therapy resulted in paracrine interaction between cancer and stromal cells likely through RAGE-HMGB1 interaction in patients with advanced prostate cancer (28). HMGB1-RAGE interactions have been examined in several prostate cancer cell lines, in hormone-refractory prostate cancer tissues, and in normal prostatic tissues (29). HMGB1 mRNA was expressed in all three cell lines, with DU145 cells, from a hormone-independent prostate cancer cell line expressing the highest level of RAGE mRNA. Untreated prostate and hormone-refractory prostate carcinomas in turn expressed higher RAGE and HMGB1 mRNA levels than normal prostatic tissue (29).

Fig. 4. Mode of cell death is important for immune cell recruitment and activation. When it is time to die, human cells have three predominant fates: apoptosis (I), autophagy (II), and necrosis (III). Upon necrotic death, HMGB1 is released into the extracellular space resulting in inflammatory cell recruitment and activation. Upon apoptotic death, HMGB1 contents are initially sequestered in apoptotic bodies in the nucleus. Later, these bodies are ingested by phagocytes and the HMGB1 is released. The exact role of HMGB1 in autophagy is unknown; however, it is hypothesized that the mode of release of HMGB1 is similar to that in apoptosis.
When coexpressed with the ligand (35), RAGE was strongly expressed in melanoma inhibitory activity PaCa-2 and PANc-1, both with high metastatic ability. On the contrary, RAGE was expressed little in BxPC-3 that has low ability.

Colon: WiDr, RKO

WiDr secreted HMGB1, showed RAGE activity. Endogenous HMGB1 was found in RKO colon cancer lines, with additional HMGB1 transfection providing additional protection against apoptosis.

Breast: MCF-7 (estrogen +)

HMGB1 is expressed at a slightly elevated level of 2-fold to 3-fold in estrogen (+) breast cancer cells. Transcription of the human HMGB1 gene in the breast cancer MCF-7 cells starts at one major site 57 nucleotides upstream from the first exon-intron boundary. The 2-fold to 3-fold increase in HMGB1 expression in breast cancer cells stimulated by estrogen may result from the action of the enhancer elements in intron 1. Several novel estrogen-tethered platinum (IV) complexes were synthesized, evaluated for their ability to up-regulate HMGB1, and screened for cytotoxicity against breast cancer cell lines. All complexes induced the overexpression of HMGB1 in estrogen receptor (+) MCF-7 cells.

Pancreatic: melanoma inhibitory activity, PaCa-2 and PANc-1 with high metastatic ability. BxPC-3 has low metastatic ability

RAGE was strongly expressed in melanoma inhibitory activity PaCa-2 and PANc-1, both with high metastatic ability. Overall, overexpression of HMGB1 protein and mRNA was measured in the malignant melanoma lines compared with NHEM.

Metastases of malignant melanoma: HTZ19d, Mel Ju, Sk-Mel-28

RAGE resulted in diminished proliferation of NCI-H358.

Lung: NCI-H358, NCI-A549, and NCI-H322

All cell lines were characterized by a low level of RAGE mRNA and lack protein expression. Overexpression of RAGE resulted in diminished proliferation of NCI-H358.

Breast cancer. Increased HMGB1 protein expression is found in primary human breast carcinomas compared with normal tissues, with HMGB1 mRNA present during mouse mammary development with low amounts measured during periods of apoptotic activity (58). As noted previously, nuclear HMGB1 facilitates DNA binding and transcriptional activity of the ERα and ERβ through enhancement of binding estrogen responsive elements (36, 60, 61). Breast cancer response to endocrine therapy does not always correlate with the quantity of expressed steroid hormone receptor (36). This lack of correlation may be due to mediation of estrogen receptor–binding to estrogen-responsive elements of target genes by HMGB1 and other adaptor proteins. Northern blot analyses of HMGB1 transcripts in breast cancer samples revealed a strong intertumoral variation, which in turn could explain varied responses among estrogen receptor–positive tumors (36).

Lung cancer. Lung carcinomas provide an exception to the “norm” of HMGB1-RAGE overexpression found within most other human tumors. Nonsmall cell carcinoma studies established a role for RAGE in the regulation of differentiation in lung tissue characterized by down-regulation of RAGE (87, 103). Loss of HMGB1-RAGE–mediated regulation of tumor cell migration and invasive processes is associated with increased aggressiveness of tumor behavior in the lung (37, 87, 103). The lung is one of few tissues (38, 87) in which RAGE

Table 3. Human cell line models of cancer and HMGB1 interactions

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Comments</th>
<th>Reference</th>
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<tr>
<td>Melanoma (primary cutaneous): Mel Im, Mel Ei, Mel Wei, Mel Ho, Mel Juso, SK-Mel-3</td>
<td>Western blotting showed up-regulation of HMGB1 in all nine cell lines versus NHEM control melanocytes. Keratinocyte staining showed increased HMGB1 staining in malignant melanoma versus NHEM controls. Overall, overexpression of HMGB1 protein and mRNA was measured in the malignant melanoma lines compared with NHEM.</td>
<td>(106)</td>
</tr>
<tr>
<td>Metastases of melanoma: HTZ19d, Mel Ju, Sk-Mel-28</td>
<td></td>
<td></td>
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<tr>
<td>Pancreatic: melanoma inhibitory activity, PaCa-2 and PANc-1 with high metastatic ability. BxPC-3 has low metastatic ability</td>
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</tr>
<tr>
<td>Colon: WiDr, RKO</td>
<td>WiDr secreted HMGB1, showed RAGE activity.</td>
<td>(32, 58)</td>
</tr>
<tr>
<td>Lung: NCI-H358, NCI-A549, and NCI-H322</td>
<td>All cell lines were characterized by a low level of RAGE mRNA and lack protein expression. Overexpression of RAGE resulted in diminished proliferation of NCI-H358.</td>
<td>(103)</td>
</tr>
<tr>
<td>Breast: MCF-7 (estrogen +)</td>
<td>HMGB1 is expressed at a slightly elevated level of 2-fold to 3-fold in estrogen (+) breast cancer cells. Transcription of the human HMGB1 gene in the breast cancer MCF-7 cells starts at one major site 57 nucleotides upstream from the first exon-intron boundary. The 2-fold to 3-fold increase in HMGB1 expression in breast cancer cells stimulated by estrogen may result from the action of the enhancer elements in intron 1. Several novel estrogen-tethered platinum (IV) complexes were synthesized, evaluated for their ability to up-regulate HMGB1, and screened for cytotoxicity against breast cancer cell lines. All complexes induced the overexpression of HMGB1 in estrogen receptor (+) MCF-7 cells.</td>
<td>(19, 124)</td>
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a possible explanation for the interference of apoptotic machinery through inhibition of the Bak pathway (30). Coexpression of RAGE and HMGB1 enhanced migration and invasion by colon carcinoma cells Colo320, DLD1, WiDr, and TCO with RAGE and HMGB1 protein and mRNA measured at similar levels (31). Colon cancer tumors expressing HMGB1 have less extensive macrophage infiltration (32), and HMGB1 derived from the WiDr cell line induces growth inhibition and apoptosis in macrophages, suggesting a role for HMGB1 in the inhibition of host immune responses within the tumor microenvironment (31). Phosphorylation levels of Rac and c-Jun-NH2-kinase/stress-activated protein kinase SAPK were increased in macrophages similarly treated with HMGB1-derived media from colon cancer cells (32).

Three nuclear matrix proteins were found to be absent in normal colonic tissue and altered in colorectal cancer: HMGB1, creatine kinase B, and heterogeneous nuclear ribonucleoprotein F (33). Enhanced expression of HMGB1 correlated with poor prognosis. RAGE expression has also been strongly associated with atypia and increased size of colorectal adenomas, colorectal metastases to lymph nodes and distal organs, and poor prognosis at any colorectal cancer stage (34). RAGE was found in 19%, 81%, and 100% of Dukes’ B, C, and D cases, respectively, correlating with invasiveness and poor outcome when coexpressed with the ligand (35).
is constitutively expressed at high levels. Soluble RAGE (sRAGE) administration does not suppress lung cancer growth and metastasis as has been found in other tumor types (87, 102). This variation may be due to the ability of sRAGE to intercept the interaction of RAGE ligands with other receptors (103). RAGE is strongly reduced at both the mRNA and protein level in nonsmall cell lung carcinomas when compared with normal lung tissues, with down-regulation of RAGE correlating with higher tumor-node-metastasis stages. This finding did not depend on the histologic subtypes; squamous cell lung carcinoma and adenocarcinoma both showed comparable down-regulation (103). Down-regulation was also observed in benign neoplasms of the lung including hamartomas. Examination of the human lung cancer line NCI-H358 in vitro and in vivo also illustrated that overexpression of full-length human RAGE is associated with diminished tumor growth with RAGE-overexpressing cells evidenced by smaller tumors in spheroid cultures in vitro and in athymic mice in vivo (103). RAGE down-regulation could be a necessary process in the formation and reorganization of tissue in lung tumors. However, the exact mechanism by which RAGE contributes to decreased tumor growth remains unclear. Several explanations have been suggested, including posttranscriptional regulation, the existence of truncated secretory isoforms, varied extracellular levels of RAGE and its ligands based on tumor location, and the activation by multiple ligands with synergistic effects between the proinflammatory and prosurvival actions (39, 40, 66, 103).

Potential Therapies Targeting HMGB1

Nonprogrammed cell death, rather than hyperproliferation, is an underexplored mechanism mediating cancer development (35). RAGE-HMGB1 signaling blockade not only tumor growth but also metastasis of C6 gliomas in immunocompetent mice supports this hypothesis (102). This finding has a wide range of implications for potential therapies based on the blockade of HMGB1-RAGE signaling. Several therapeutic strategies have been suggested based on the properties of HMGB1, RAGE, and their interactions: (a) the administration of sRAGE, (b) antibodies to RAGE or TLR2 receptors or to the ligand (HMGB1/amphoterin), (c) administration of the inhibitory A box of HMGB1 which antagonizes the B box functional activity, (d) or small molecule targeting of signaling molecules (Fig. 5; refs. 35, 41). Manipulation of ERK, signal transducers and activators of transcription, myeloid differentiation protein 88, IL-1 receptor-associated kinase, or other pathways involved in promoting inflammation after HMGB1 signaling are also possibilities (Table 4; ref. 35). Further investigation is needed to dissect the extent of extracellular activities of HMGB1 in cancer and the proteins it interacts with to more fully understand these therapies and their implications in cancer therapeutics.

HMGB1-neutralizing antibodies. Administration of anti-HMGB1 antibody before and shortly after endotoxin exposure increases the survival of exposed mice (9). This response is dose dependent, with a higher survival rate correlating with increased frequency of administration of the anti-HMGB1 antibody (9). Neutralizing anti-HMGB1 antibodies inhibit release of TNF-α and IL-6 by blocking extracellular HMGB1 but does not prevent HMGB1 secretion. Ischemic insult-causing hepatic injury is preventable in mouse models by treatment with anti-HMGB1 antibodies, most likely through TLR4-mediated inflammatory suppression (15, 68). The role for NF-κB in ischemia-reperfusion induced apoptosis in the liver and its interaction with HMGB1 in this circumstance is not currently established. However, inhibition of NF-κB after hepatectomy results in massive apoptosis, impaired liver function, and decreased survival (15, 42). Because of response

![Fig. 5. Possible therapeutic strategies targeting HMGB1. Several therapies have been suggested based on the properties of HMGB1, RAGE, and their interactions. Extracellular HMGB1 can be blocked by administration of sRAGE, antibodies to the RAGE or TLR2 receptors or the ligand (HMGB1/amphoterin), or administration of the inhibitory A box of HMGB1 which antagonizes the B box functional activity (35, 41). Intracellular HMGB1 can be blocked at the nuclear level by platinum therapy. At the cytosolic level, HMGB1 can be blocked from entering the extracellular space by ethyl pyruvate, acetylcholine/nicotine, or stearoyl LPC. By blocking HMGB1 at any one of these locations, further release of inflammatory mediators or tissue repair cytokines can potentially be inhibited. Modified from Cytokine Growth Factor Review (15), with permission from Elsevier.](image_url)
HMGB1 and Cancer

Table 4. Therapeutic strategies

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Prototypes and advantages</th>
<th>Disadvantages</th>
<th>Selected references</th>
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<tbody>
<tr>
<td>HMGB1 sequestration</td>
<td>Platinum therapy: sequesters HMGB1 into the nucleus in apoptotic death</td>
<td>Myeloid suppression, ototoxicity, nephrotoxicity</td>
<td>(122, 123)</td>
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</table>
| Targets of Extracellular HMGB1 | A box HMGB-1: antagonistic, is more efficacious than polyclonal HMGB-1 antibodies in sepsis and arthritis  
Anti-HMGB-1 Ab: inhibits TNFα, IL-6, increases shock survival rate | A box HMGB-1: short half life                                                | (9, 110)            |
| Targeting receptors          | sRAGE: inhibits tumor proliferation, invasion and expression of MMPs, blocks endogenous tumor growth  
Stearoyl LPC: inhibits HMGB1 secretion from macrophages and protects mice from lethal sepsis  
Acetycholine/nicotine: like stearyl LPC | Anti-HMGB-1 Ab: does not prevent HMGB 1 secretion  
sRAGE: has shown promise in animal models only thus far. Stearoyl LPC; mechanism of action unknown  
Stearoyl LPC: exact mechanism of action remains unknown | (43, 69) |
| Inhibition of HMGB1 release  | Ethyl pyruvate: inhibits the release of TNF and HMGB-1, attenuates activation of p38 and NF-κB | Unknown efficacy outside of septic shock models                               | (46)                |

NOTE: Several therapies have been suggested for inflammatory diseases and cancer that rely on properties of HMGB1 and RAGE. These therapies are grouped according to their ability to sequester HMGB1, target extracellular HMGB1, target receptors such as RAGE, or inhibit HMGB1 release. These therapies vary in their efficacy and availability for human administration.

variability from NF-κB blockade, more consistent outcome may be obtained by blocking initial HMGB1 secretion rather than targeting its presence in the extracellular space.

sRAGE and RAGE-HMGB1 blocking strategies. Blockade of RAGE signaling pathways could also result in attenuation of tumor development and growth. Several strategies that block HMGB1-RAGE signaling have been reported, such as the administration of extracellular ligand binding domain of sRAGE, administration of blocking Fab fragments derived from anti-RAGE and/or anti-HMGB1 IgG, and generation of stably transfected C6 glioma expressing sRAGE (15). sRAGE in particular acts as a decoy to prevent RAGE signaling and has been used successfully in animal models (43). Human sRAGE generated by alternative splicing of the RAGE transcript is able to bind heparin, enabling distribution in the extracellular matrix and at the cell surface (43). Human sRAGE can also be influenced by proteinases similar to sRAGE found in mice (43). HMGB1-RAGE signaling inhibition suppresses activation of p44/p42, p38, SAP/MAP kinases, and NF-κB (39, 43–45, 84), along with molecular effectors linked to tumor proliferation, invasion, and expression of MMPs (15). sRAGE administration blocks endogenous growth of many tumors, including murine papillomas (102).

Ethyl pyruvate. Ethyl pyruvate inhibits the release of TNF and HMGB1 from endotoxin-stimulated RAW 264.7 murine macrophages, as well as attenuates activation of both the p38 mitogen-activated protein kinase and NF-κB signaling pathways. Ethyl pyruvate treatment of septic mice decreased circulating levels of HMGB1 (46). Pretreatment with ethyl pyruvate also prevented endotoxin lethality and inhibited the release of TNF and HMGB1 (46). Despite these findings, the exact mechanism of action of ethyl pyruvate as a HMGB1 inhibitor is unknown and its use is currently being explored in murine inflammatory bowel disease models.³

Truncated forms of HMGB1. Structural characteristics of HMGB1 revealed an “A box” with possession of antagonistic properties to the full length or the agonistic B box of HMGB1 (Fig. A; refs. 25, 110). To capitalize on this function, truncated forms of recombinant HMGB1 containing the A box were tested and found to have antagonistic properties (111). A-box treatment has been evaluated in a sepsis and arthritis model and found to be equally efficacious as treatment with polyclonal anti-HMGB1 antibodies; however, the short half-life of A box has compelled the need for structural modifications (25, 111, 112). A-box therapy has not been tested in murine tumor models.

Stearoyl (18:0) lysophosphatidylcholine. Stearoyl (18:0) lysophosphatidylcholine has potential as a therapeutic agent for use in systemic inflammatory states due to its ability to inhibit HMGB1 secretion from activated macrophages and protect mice from lethal sepsis (69, 113, 114). Lysophosphatidylcholine treatment of activated macrophages abolished HMGB1 cytoplasmic accumulation and subsequent extracellular secretion (115). Lysophosphatidylcholine binds to the G protein–coupled G2A receptor to increase intracellular calcium ions; however, the exact mechanism of action remains unknown (16, 69, 116).

α7-Nicotinic acetylcholine receptor agonists. The nervous system actively regulates innate immune responses and macrophage production of inflammatory cytokines (69). Vagus nerve activation inhibits macrophage release of HMGB1 (69, 117). Acetylcholine could itself be responsible for the antiinflammatory mechanism. Indeed, acetylcholine was the first inhibitor of HMGB1 secretion from macrophages to be described (69, 115, 118). This finding led to the use of nicotine to activate the

³ S. Davet, M.T. Lotze, and S.E. Plevy, manuscript in preparation.
α7-nicotinic acetylcholine receptor, the receptor responsible for the modulation of antiinflammatory effects (69, 119). Macrophage treatment with nicotine showed inhibition of HMGB1 secretion and prevented activation of the NF-κB signaling pathway (69, 115, 118). Mice exposed to septic conditions possessed reduced HMGB1 levels in serum and improved survival after nicotine therapy (69, 115). Currently, nicotine therapy is not feasible, considering its potential toxicity and nonspecific receptor activation. However, an agonist of the α7-nicotinic acetylcholine receptor with increased specificity could present as an alternative therapy (69).

**Platinum therapy.** Platinum therapy characteristically creates two adducts: a 1,2 intrastrand (dGpG) crosslink and a minor 1,3 intrastrand (dGpTpG) adduct (62). Both of these adducts are repairable by an excision repair system. HMG domain motifs bind specifically to the major platinum DNA dGpG adducts, creating a shield against the human excision nucleases accomplished through the acidic domain (120, 121). In this role, cisplatin serves as a sequestration mechanism for HMGB1 and induced ototoxicity in rats (122). The increase in HMGB1 was prevented by the administration of a known protective agent against cisplatin toxicity, the L-methionine (122, 123), suggesting that L-methionine might also block HMGB1-activities.

**Conclusions**

Cancer evolution enables poseurs of cell damage to promote reparative angiogenesis, stromagenesis, and epithelial proliferation in a grand masquerade party, with many of the involved players not yet unmasked. Even inflammatory cells including PDCs and macrophages that have been identified within tumors wear many masks with some identified roles and others that are still a mystery. HMGB1 is itself the great masquerader—it is puzzling how one protein could be responsible for so many roles, both within and outside cells. HMGB1 clearly plays a role in cancer development and metastasis, with RAGE-HMGB1 signaling promoting spread of most tumor types. However, the full extent of that role remains cryptic along with the possibility of its interaction with other signaling systems. The development of new therapies will aid in the process of uncovering the extent of HMGB1 involvement in the numerous cellular processes in which it is hypothesized to be involved, including cancer.

**Note Added in Proof**

At the Death, Danger and Immunity Meeting held at the Institut Pasteur, March 8 to 9, 2007, Laurence Zitvogel addressed the question of whether TLR signaling plays a role in immunogenic cell death. Dr. Zitvogel presented data that the response to an irradiated ovalbumin expressing EL4 lymphoma was TLR4 and MyD88 dependent, but without an apparent role for TLR2/TLR7/TLR9/TRIF. Interestingly, large amounts of HMGB1 could be identified in the supernatants of various tumor cell lines treated with doxorubicin or radiation. Dr. Zitvogel showed that HMGB1 binds to TLR4 using pull-down immunoblots from dying cells. In addition, Dr. Zitvogel was able to show that in breast cancer patients with the AspGly299 TLR4 polymorphisms, the time to progression was faster in those without the mutated allele. Thus, normal TLR4 is protective, presumably because of its ability to respond more rapidly to the DAMP, HMGB1.

**Acknowledgments**

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43. Tanuma S, Yagi T, Johnson GS. Endogenous ADP-ribosylation of high mobility group h protein 1 and 2 and histone H1 following DNA damage in intact cells. Arch Biochem Biophys 1985; 237:38 –42.


57. Tanuma S, Yagi T, Johnson GS. Endogenous ADP-ribosylation of high mobility group h protein 1 and 2 and histone H1 following DNA damage in intact cells. Arch Biochem Biophys 1985;237:38 –42.


