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Redox Modulation of HMGB1-Related Signaling

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Abstract

Significance: In the cells' nuclei, high-mobility group box protein 1 (HMGB1) is a nonhistone chromatin-binding protein involved in the regulation of transcription. Extracellularly, HMGB1 acts as a danger molecule with properties of a proinflammatory cytokine. It can be actively secreted from myeloid cells or passively leak from any type of injured, necrotic cell. Increased serum levels of active HMGB1 are often found in pathogenic inflammatory conditions and correlate with worse prognoses in cancer, sepsis, and autoimmunity. By damaging cells, superoxide and peroxynitrite promote leakage of HMGB1. **Recent Advances:** The activity of HMGB1 strongly depends on its redox state: Inflammatory-active HMGB1 requires an intramolecular disulfide bond (Cys23 and Cys45) and a reduced Cys106. Oxidation of the latter blocks its stimulatory activity and promotes immune tolerance. **Critical Issues:** Reactive oxygen and nitrogen species create an oxidative environment and can be detoxified by superoxide dismutase (SOD), catalase, and peroxidases. Modifications of the oxidative environment influence HMGB1 activity. **Future Directions:** In this review, we hypothesize that manipulations of an oxidative environment by SOD mimics or by hydrogen sulfide are prone to decrease tissue damage. Both the concomitant decreased HMGB1 release and its redox chemical modifications ameliorate inflammation and tissue damage. *Antioxid. Redox Signal.* 20, 1075–1085.

Introduction

THE DISCOVERY OF the high-mobility group (HMG) proteins as nuclear factors (NFs) was more than 30 years ago when the proteins were named for their high mobility in electrophoretic polyacrylamide gels (28). High-mobility group box protein 1 (HMGB1) is an evolutionary-conserved ubiquitously expressed member of the HMG family. Structurally, HMGB1 is a single polypeptide chain of 215-amino-acid length that contains two positively charged DNA-binding motifs, HMG-box A (1–79) and B (89–163), and an acidic C-terminus (186–215) with high amounts of aspartic or glutamic acid residues (80) (Fig. 1).

HMGB1 is a protein with various functions, depending on its location outside or inside the cell. In the nucleus, it acts as a nonhistone architectural chromatin-binding factor (78). It binds to DNA in the minor groove without a sequence specificity and induces DNA bending, thereby participating in transcriptional regulation (5). HMGB1 is expressed by almost all cells, except those without the nucleus, for example, erythrocytes. HMGB1

either can be secreted by activated macrophages, dendritic cells, and natural killer cells or can passively leak from necrotic or injured cells (45). Distress, especially oxidative stress, induces the release of HMGB1 (76, 81). For migration out of the nucleus, HMGB1 is acetylated near its nuclear-localization sequences, thus blocking the interaction with the nuclear importer and inhibiting re-entry to the nucleus (45). Acetylated cytosolic HMGB1 moves to cytoplasmic secretory vesicles that allow the regulated secretion of the protein (27). Extracellular HMGB1 can act as a proinflammatory mediator, whose secretion is delayed compared to the classical early proinflammatory cytokines (95). HMGB1 signals through Toll-like receptor (TLR) 4 and the receptor for advanced glycation endproducts (RAGE) activating NF- κ B signaling (54).

Extracellular HMGB1 forms complexes with various molecules and, consequently, interacts with a plethora of cell surface receptors (Fig. 2) (65). Extracellular HMGB1 provokes the production of proinflammatory cytokines, cell proliferation, and cell migration and can thus be considered a proinflammatory cytokine (4, 15).

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FIG. 1. Structure of HMGB1. High-mobility group box protein 1 (HMGB1) consists of 215 amino acids organized into two HMG boxes, which are the DNA-binding domains, and an acidic C-terminus.

Release of HMGB1 from Apoptotic and Necrotic Cells

When a cell undergoes necrosis, its membrane loses its integrity. Intracellular molecules rapidly leaking from necrotic cells act as endogenous adjuvants and foster inflammation (55). HMGB1 has the features of an intracellular death-associated molecular pattern (DAMP): it is a ubiquitous molecule, but in healthy conditions not being detectable extracellularly. Generally, HMGB1 is not tightly bound to chromatin, and therefore it passively diffuses from necrotic cells (Fig. 3). During apoptotic cell death, under-acetylation of histones and chromatin condensation cause irreversible attachment of HMGB1 to the chromatin. In contrast, the acetylation status of HMGB1 itself is not changed during apoptosis. Consequently, in apoptotic cells, HMGB1 is sequestered inside the nucleus, thus contributing to the anti-inflammatory response exerted by these cells under physiological conditions (60). Here apoptotic cells are swiftly cleared before their membrane integrity breaks down. In conditions of clearance deficiency, they will persist and undergo secondary necrosis, where most of the HMGB1 is frozen to nucleosomes inside the dying cell (60). However, a fraction of nucleosomes with HMGB1 piggyback is released from secondary necrotic cells frequently seen in the tissues of patients suffering from systemic lupus erythematosus (SLE) (83) (Fig. 3). Despite usually being poorly immunogenic (29), nucleosomes and their constituents (DNA and histones) become the targets of the hallmark autoantibodies characterizing SLE (35, 50, 51); the HMGB1–nucleosome complexes reportedly promote inflammation and autoimmunity *via* TLR 2 (83).

Complex Relations of Reactive Oxygen Species/Reactive Nitrogen Species and HMGB1

During physiological processes, living cells constantly generate low levels of reactive oxygen species (ROS) as the consequence of aerobic metabolism, with mitochondria being the major site of ROS production. Beside ROS, cells produce reactive nitrogen species (RNS). Intracellular accumulation of ROS and RNS can be triggered by both exogenous and endogenous factors as irradiation, inflammation, environmental toxins, cigarette smoke, or air pollution (64). ROS/RNS can cause damage to all biomolecules (proteins, lipids, and DNA) and ultimately lead to cell death, being implicated in the etiology of several pathologies (64). Nonenzymatic antioxidants and antioxidant enzymes such as catalase, glutathione peroxidase, thioredoxin reductase, and superoxide dismutase (SOD) neutralize ROS and RNS before damage of cell organelles occurs (53) (Fig. 4). During apoptosis, increased levels of ROS/RNS have been reported (10).

The complex relations of ROS/RNS and HMGB1 can be best demonstrated by the fact that ROS/RNS have been suggested to be both the cause and consequence of HMGB1 release. Namely, Tsung *et al.* showed that hypoxic cultured hepatocytes release HMGB1 through an active process facilitated by TLR4-dependent ROS production (81). In this case, ROS induce HMGB1 release through calcium/calmodulin-dependent kinase-mediated signaling. They demonstrated that antioxidants reduce HMGB1 release and tissue damage in the liver ischemia–reperfusion.

Oxidative/nitrosative stress from the massive generation of ROS/RNS is the major pathophysiological mechanism for

FIG. 2. HMGB1-associated molecules and interactions with cell surface receptors. Beside HMGB1 signalling *per se* employing receptor for advanced glycation endproducts (RAGE) and Toll-like receptor 4 (TLR4), HMGB1 forms complexes with immunostimulatory molecules as the TLR4 ligand lipopolysaccharide (LPS), the TLR2 ligands Pam₃CSK₄ and nucleosomes, the IL-1R ligand IL-1 β , the CXCR4 ligand CXCL12, as well as the TLR ligands RNA and DNA. HMGB1 has also been described to bind to CD24 and thrombospondin, eliciting negative regulatory signals.

FIG. 3. Passive release of HMGB1 from dying and dead cells. In viable cells, HMGB1 acts as nonhistone, architectural chromatin-binding factor. During apoptosis under-acetylation of histones leads to chromatin condensation, and HMGB1 is irreversibly attached to the chromatin. If apoptotic cells are not cleared in time by phagocytes, they undergo secondary necrosis, and HMGB1-nucleosome complexes can leak through the disrupted plasma membrane. Primary necrotic cells release free HMGB1.

cell death in myocardial ischemia and reperfusion (53). In the absence of extracellular antioxidants, peroxynitrite is a major oxidant, and exposure to it has been shown to cause myocardial damage in the isolated rat hearts, anesthetized rats, and other pathological models of myocardial dysfunction in several species *in vivo* or *ex vivo* (17). Myocardial necrosis results in an increased HMGB1 release in the myocardium with severe proinflammatory effects (46). In line with this finding, HMGB1 accumulation has been associated with inflammatory responses in the heart (87), where HMGB1 signals through RAGE and TLR4, expressed by cardiac myocytes and non-myocytes (46). Loukili *et al.* reported that peroxynitrite promotes significant release of

HMGB1 in H9c2 cardiomyoblasts and in primary murine cardiac cells *in vitro* (46). In this study, they also used two different peroxynitrite metalloporphyrin decomposition catalysts to significantly lower down the levels of HMGB1 and to reduce the myocardial infarct size after ischemia-reperfusion in rats. Mild ROS production is important for stimulating the translocation of HMGB1 from the nucleus to the cytoplasm and subsequent activation of autophagy in cultured mouse embryonic fibroblasts (73). Thus, HMGB1 is necessary for the activation of autophagy in response to oxidative stress and serves as a redox sensor. In these studies, H_2O_2 or knockdown of SOD-1 increased the release of HMGB1.

FIG. 4. Development of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Leakage of electrons from the mitochondrial electron transport chain (ETC), NAD(P)H oxidase, and xanthine oxidase, and uncoupling of nitric oxide (NO) synthases lead to the development of $O_2^{\bullet-}$. Peroxynitrite can be produced from NO and $O_2^{\bullet-}$. ROS can be detoxified by catalase, peroxidase, and superoxide dismutase. Free ROS/RNS cause oxidative damage of DNA, proteins, and lipids, resulting in the loss of the mitochondrial membrane potential ($\Delta\psi_m$), in apoptosis or necrosis.

On the other side, numerous studies reported a dramatic increase of ROS as a consequence of HMGB1 release, with particular emphasis on peroxynitrite formation. In the study by Sappington *et al.*, the authors demonstrated that HMGB1 causes alteration of the gut barrier by increasing the inducible nitric oxide synthase (iNOS) expression and subsequently peroxynitrite formation in Caco-2 human enterocytic monolayers, an effect that was blockable by the use of NO, superoxide, or ONOO⁻ scavengers (59). This increase of ROS formation can be related to the effect that HMGB1 has on gene and protein expression of NAD(P)H oxidase. For example, in hemorrhagic shock/resuscitation, HMGB1 plays an important role in activating the TLR4 signaling pathway with overexpression of NAD(P)H oxidase in neutrophils *ex vivo*, leading to sustained overproduction of superoxide and ROS (16).

From all these studies, it is obvious that superoxide and peroxynitrite play important roles, and that manipulation of their generation could (i) decrease tissue damage and HMGB1 release or/and (ii) modulate further inflammation and tissue damage due to inflammatory responses induced by released HMGB1.

HMGB1 Involved in Diseases

Increased concentrations of extracellular HMGB1 have been reported in various pathological conditions such as sepsis (murine model) (95), infections (*in vitro*) (37), arthritis (clinical research) (77), and cancer (clinical research) (92). After lipopolysaccharide (LPS) stimulation, HMGB1 was released by cultured macrophages, and also serum levels of HMGB1 were significantly increased *in vivo*, leading to the death of mice after systemic LPS injection. Consequently, exposure of mice to HMGB1 was also lethal (88). Under conditions of chronic inflammation like rheumatoid arthritis, increased cytoplasmic and extracellular HMGB1 expression has been observed in the human synovial fluids, whereas in healthy persons, HMGB1 expression was limited to the nuclei (77).

Elevated levels of serum and plasma HMGB1 circulate in patients with SLE and in murine lupus models; in the former, HMGB1 levels correlated with the SLE disease activity index (1, 36, 83). In SLE, patients with myositis, vasculitis, renal involvement, or skin lesions displayed increased amounts of circulating HMGB1 (1). Overexpression of HMGB1 and its receptor RAGE has been associated with cancer progression, invasion, and metastasis of many tumor types, including cancers of the breast (clinical research) and the colon (clinical research), and in melanoma (cell lines) (14).

These findings highlight the central role of HMGB1 in several pathological conditions. HMGB1 may, therefore, qualify as a target for therapeutic interventions. Treatment of arthritis-prone mice with a truncated A box, a competitive HMGB1 antagonist, or monoclonal and polyclonal anti-HMGB1 antibodies showed beneficial therapeutic effects (61).

SOD Mimics as Potential Therapeutics to Reduce Oxidative Stress, Cell Death, and Concomitant HMGB1 Release: The Lack of Selectivity Is an Advantage

Pyruvate, an intermediate in the glucose metabolism, has potent nonenzymatic antioxidant properties and serves as free radical scavenger (24). It detoxifies peroxides, peroxynitrite, and hydroxyl radicals (12, 96). Pyruvate and ethyl pyruvate both inhibit the HMGB1 secretion (82) and show

protective effects in animal models of reperfusion injury (32), sepsis, hemorrhagic shock (6), and ischemia (57). Ulloa *et al.* suggested that the inhibition of signaling through NF- κ B and/or the p38 MAPK pathway may underlie the regulation of HMGB1 release from LPS-stimulated macrophages (82). Recently, in their study, Jang *et al.* investigated the molecular anti-inflammatory mechanism of ethyl pyruvate. They found that ethyl pyruvate induced heme oxygenase-1, mediated through the p38 MAPK and Nrf2-dependent pathway by decreasing cellular glutathione levels in RAW 264.7 cells (34). Ethyl pyruvate significantly inhibited the LPS-stimulated iNOS expression and HMGB1 release in RAW 264.7 cells. In septic mice, ethyl pyruvate decreased serum HMGB1 and increased survival (34). Beside ethyl pyruvate, several other agents with antioxidative properties as quercetin, green tea, N-acetylcysteine, and curcumin showed anti-inflammatory effects by inhibiting the release and the cytokine activities of HMGB1 (74).

The role of SOD mimics could have beneficial effects on both, HMGB1 release and its immunogenic effects. Indeed, one study dealing with the effect of manganese SOD and copper/zinc SOD overexpression (41) *in vitro* showed suppression of multicellular tumour spheroid growth through inhibition of ROS induced necrosis and HMGB1 release. Conversely, SOD mimics and peroxynitrite decomposition catalysts have been used mostly as a tool to demonstrate the essential role of ROS/RNS in HMGB1 release, but never really studied as potential therapeutics from that perspective. Superoxide is referred to as a member of ROS, acting as a reducing agent in its anionic state, and as an oxidant in its protonated form (33). Although being a radical, superoxide is not a highly reactive initiator of free radical reactions (33). One of the main reasons for its toxicity is, in fact, its reaction with NO. NO has evolved from a signaling molecule that regulates blood pressure to a molecule implicated in almost all metabolic pathways, as extensively reviewed elsewhere (47). These roles NO maintains by reacting with metal centers (9), by modulating thiol groups of proteins (forming S-nitrosothiols or/and disulfides) (25, 30), or/and by reacting with superoxide to form peroxynitrite (18, 68, 79). While the first two represent the physiological mechanisms of action, the latter mechanism is characteristic for pathological processes.

SODs usually keep the level of superoxide below 100 pM under physiological conditions (33). NO is rapidly removed by its diffusion through tissues into erythrocytes where it is converted to nitrate after reaction with oxyhemoglobin, limiting the biological half-time (53). When the enzyme activity of SOD is impaired or the enzyme level is insufficient, peroxynitrite formation increases (11). When superoxide and NO meet, they will spontaneously form the much more powerful oxidant peroxynitrite by a diffusion-limited reaction (53). In conditions of inflammation, the synthesis of superoxide and NO and the formation to peroxynitrite can be strongly enhanced (53). Although peroxynitrite has a short half-life (~10–20 ms), it can cause oxidative damage by interaction with proteins (nitration, oxidation, and aggregation), lipids (nitration and oxidation), and DNA (oxidation) (53, 68, 79). The formation of peroxynitrite and its extensive reactions with biomolecules is implicated in inflammatory responses and inflammation-related diseases. The design of the therapeutics against the latter caused by peroxynitrite is trichotomous: (i) removal of superoxide (48), (ii) inhibition of iNOS (42, 85), and, recently, (iii) scavenging of peroxynitrite (68, 79).

Three particular classes of SOD mimics have been described in the literature as promising therapeutics that have entered different phases of clinical trials: manganese salen derivatives, manganese(III) porphyrinato-complexes, and manganese(II) penta-azamacrocyclic complexes, as recently reviewed (3, 33).

The main targets of successfully designed SOD mimics are said to be inflammation-based diseases governed by overproduction of superoxide. SOD mimics would thus remove superoxide, producing H_2O_2 , or remove both (like in the case of salen complexes) (3, 33). This way, the role of NO is preserved, and subsequent formation of peroxynitrite is prevented (which can additionally be removed by porphyrin-type of SOD mimics) (3, 19, 68). Because of this main dogma, only few studies tested a direct reaction of the SOD mimics with NO (20, 21, 56, 63). Since our previous findings showed that manganese-SOD could react with NO through a dismutation mechanism (23), we studied in detail the reaction of penta-azamacrocyclic SOD mimics with NO *in vitro* (20) and on cellular models (21). We observed that these complexes catalyze NO dismutation, that is, generation of reactive nitrosonium (NO^+) and nitroxyl (NO^-/HNO) species; the former being able to cause S-nitrosylation of protein thiols and the latter their oxidation (66).

SOD mimics did not change the cell morphology or survival (21). Surprisingly, however, the treatment with SOD mimics completely abrogated the release of NO and peroxynitrite from the cells, while the protein and mRNA levels of iNOS remained unchanged. As of yet, this is the only chemical compound with a promising pharmacological effect that has been reported to remove both superoxide and NO, transforming them into more benign species without altering the enzymatic pathways for their production. This also questions the need for peroxynitrite scavengers when, in fact, this class of complexes efficiently prevents generation of peroxynitrite and moderately reacts with it. In the light of the above-mentioned tightly linked ROS-RNS-induced HMGB1 release and/or HMGB1-induced ROS/RNS generation, it is tempting to speculate that the use of this class of mimics would significantly alter the level of all, superoxide, NO, and peroxynitrite, switching the cells to survival or apoptosis rather than to necrotic cell death.

Hydrogen Sulfide: A Scavenger of Peroxynitrite?

Recent studies showed that hydrogen sulfide (H_2S) plays an important role as a signaling molecule regulating blood pressure (52, 91) and ameliorating ischemia-reperfusion injury (7, 39, 43, 67) and inflammation (58, 67, 90). Inflammation

is particularly interesting, as the results are quite contradictory. One is obvious; in most inflammatory disease models (rheumatoid arthritis and septic shock), H_2S levels are increased (67, 90). It is not clear whether this is a defense mechanism against oxidative stress induced by immune stimulation or whether H_2S promotes further immune stimulation. Whiteman *et al.* (89) originally suggested that H_2S could scavenge peroxynitrite, but a recent kinetic *in vitro* study by Carballal *et al.* (8) strongly argued that based on the kinetic parameters, H_2S cannot compete with much more abundant intracellular thiols, such as glutathione.

We recently questioned this finding both mechanistically and physiologically showing that even at low micromolar concentrations, H_2S could prevent peroxynitrite-induced cell death (22). Further, peroxynitrite-induced DNA damage often results in activation of poly[ADP-ribose] polymerase 1 (PARP-1), which initiates repair of chromatin damage (53). After PARP-1 activation, high amounts of ADP-ribose are generated, which leads to inactivation of ATP-binding cassette (ABC) transporter (13). The addition of H_2S to the cell culture medium partially prevented ABC transporter inactivation. Finally, nitration of the proteins was almost abolished by the presence of H_2S . The reaction of H_2S with peroxynitrite was additionally interesting, as it leads to the generation of $HSNO_2$, a molecule that can spontaneously decompose and release NO (22).

This discrepancy between *in vitro* kinetic data, which favors the reaction of peroxynitrite with glutathione, and obvious strong protective effects of H_2S *in vivo*, could be explained by much higher, unconstrained diffusibility of H_2S compared to larger molecules. In addition, gaseous H_2S is shown to disappear very fast from plasma presumably due to electrostatic interactions of the H_2S anion with the positively charged areas of proteins. Overall, these data suggest a strong antioxidant capacity of H_2S in preventing tissue damage, cell death, and possibly HMGB1 release. Importantly, H_2S could also affect the redox status of cysteine residues, which, as a hypothesis, we address further in the following section.

Oxidation of HMGB1 Regulates Cytokine Function

HMGB1 contains three conserved cysteines, which are sensitive to oxidation: Cys23, Cys45 (Box A), and Cys106 (Box B) (31). It has been reported that the activity of HMGB1 strongly depends on the redox state of these cysteine residues (70). Early studies suggested that in mild oxidative conditions, Cys23 and Cys45 readily form an intramolecular disulfide bridge, whereas Cys106 remains in the reduced state (31) (Fig. 5). The disulfide bond between Cys23 and Cys45 could

FIG. 5. Oxidative states of HMGB1. In inflammatory conditions, Cys23 and Cys45 form an intramolecular disulfide bond, whereas Cys106 remains in the reduced state (HMGB1 is semioxidized). Oxidation of HMGB1 by ROS/RNS abolishes its proinflammatory features.

be broken by glutaredoxin-catalyzed GSH-dependent reduction. This study suggested that mutation of Cys106 impairs the nuclear distribution and is critical for nucleoplasmic shuttling of HMGB1. The role of Cys106 was further confirmed by Tang *et al.* (72). The authors showed that mutation of Cys106, but not vicinal Cys23 and Cys45, promotes cytosolic localization and sustained autophagy while the intramolecular bridge Cys23–Cys45 is required for binding to Beclin1 and sustained autophagy. In another study, the same group demonstrated that the Cys106Ala mutant has decreased autophagy compared with wild-type HMGB1 with Cys106 in a reduced state (71). In contrast, oxidized HMGB1 leads to activation of caspase-3 and caspase-9 and the induction of the mitochondrial pathway of apoptosis. The results also suggested that Cys106 is required for HMGB1 binding to TLR4 and activation of cytokine release in macrophages.

Oxidation of HMGB1 cysteine residues has been detected during cell death (84). The activation of caspases during apoptosis leads to the production of ROS in the mitochondria, which subsequently oxidizes the potentially dangerous HMGB1. Kazama *et al.* has shown that oxidation of HMGB1 is sufficient to block its stimulatory activity and to promote immune tolerance (38). Conversely, it has been speculated that oxidized and reduced forms of HMGB1 might differentially bind to their receptors (84), since it has been shown that oxidation-specific epitopes *e.g.*, oxidized low-density lipoproteins are recognized by pattern recognition receptors (*e.g.*, TLR4) of immune cells (49). Additionally, oxidation of HMGB1 might generate neo-epitopes, which foster the development of autoantibodies as detected in autoimmune diseases (40).

The most recent detailed mass spectrometric analysis coupled with functional studies of various oxidative modifications of HMGB1 cleared up some of the hanging questions about HMGB1 activity. Using tandem-mass spectrometric analysis, Yang *et al.* showed that both Cys106 in the reduced form and the Cys23–Cys45 disulfide bond are required for HMGB1 to induce nuclear NF- κ B translocation and tumour necrosis factor production of macrophages. Both irreversible oxidation to sulfonates and complete reduction to thiols of these cysteines inhibited cytokine production. Using acetaminophen to induce hepatic necrosis, they observed that during inflammation, the predominant form of serum HMGB1 is the active one, (Cys106 thiol and Cys23–Cys45 disulfide), whereas the inactive, oxidized form of HMGB1 accumulates during resolution of inflammation and hepatic regeneration (94). The oxidation of HMGB1, therefore, serves as a feedback mechanism to control the proinflammatory activity of HMGB1 *in vivo* (94) (Fig. 6).

Recently, Venereau *et al.* confirmed the findings by Yang *et al.* and additionally described the influence of redox modifications on the chemotactic activity (86). They showed that only the fully reduced form of HMGB1, where all cysteines are in the thiol state, can recruit motile cells, whereas terminal oxidation to sulfonates abrogates the cytokine-inducing and chemoattractant activity of HMGB1. Since the disulfide and thiol states of cysteines in HMGB1 are mutually exclusive, these different HMGB1 states orchestrate either leukocyte recruitment or activation of cytokines, respectively (86).

The recruitment of inflammatory cells to damaged tissues and the induction of cytokines by different states of HMGB1

FIG. 6. Double-edged role of ROS/RNS in HMGB1 release and resolution of inflammation. On one hand, ROS/RNS induce cell death leading to the leakage of proinflammatory HMGB1, which further enforces cell death in the environment. On the other hand, the oxidation of HMGB1 by ROS/RNS into its inactive form acts as a feedback mechanism to control its proinflammatory activity.

involve different receptors (62) (Fig. 7). Disulfide HMGB1 promotes cytokine release *via* its interaction with the TLR4, whereas thiol HMGB1 forms a heterocomplex with the chemokine CXCL12 that acts exclusively *via* CXCR4 and not *via* other HMGB1 receptors (62). Reduced HMGB1 binds to RAGE, but not to TLR4, and promotes Beclin1-dependent

FIG. 7. Functions and receptors of HMGB1 are dependent on its redox state. HMGB1 with C23 and C45 in a disulfide bond signals *via* TLR4, eliciting the release of proinflammatory cytokines. Complete reduction of cysteines to thiols abrogates the cytokine-stimulating activity; it induces autophagy *via* RAGE. Thiol HMGB1-complexed CXCL12 serves as a chemoattractant and induces cell migration. The actions of disulfide and thiol HMGB1 are mutually exclusive. Terminal oxidation of cysteines to sulfonates completely abolishes HMGB1 activity and contributes to the resolution of inflammation.

autophagy (75). On the other hand, oxidized HMGB1 induces apoptosis *via* a caspase-9/3 intrinsic pathway (71).

Detection of HMGB1

HMGB1 levels have been shown to be an important biological marker in a plethora of pathogenic inflammatory conditions. Increased serum levels of HMGB1 often correlate with a worse prognosis, for example, in cancer, sepsis, and autoimmunity (77, 88, 92). Therefore, a reliable assay to quantify HMGB1 in serum and plasma is mandatory. Usually, ELISA, immunoblots, and EMSA are used to detect HMGB1. In this section, we want to highlight the possible problems emerging in the analyses of HMGB1.

Occurrence of HMGB1 in various redox states

Detection of HMGB1 is problematic since it occurs in various redox states, and detection antibodies may be specific for only one form. Urbonaviciute *et al.* reported that HMGB1 is not detectable in lysates from late-apoptotic Jurkat cells by immunoblot analysis, employing an antibody specifically detecting the reduced form of the molecule (84). Consequently, reduction of HMGB1 with β -mercaptoethanol or glutathione enabled detection. In contrast, HMGB1 in the lysates of viable cells was detected under nonreducing conditions. Since detection of oxidized HMGB1 released from apoptotic cells may fail because of the usage of a not suitable detection antibody, it is essential to check which specificities of HMGB1 are identified by the respective assay agents. In this context it is essential to mention that HMGB1 is a highly evolutionarily conserved protein with 99% sequence homology among all mammals (80). Only two residues out of its 215 amino acids are substituted in the rodent and human versions. Therefore, immunization of animals with HMGB1 as an immunogen might be difficult and especially create antibodies directed against the non-native forms of HMGB1.

Complex formation of molecules with HMGB1 interferes with HMGB1 detection

It has been described that results of HMGB1 ELISA in plasma/serum do not correlate with those of immunoblotting (26). The formation of complexes of HMGB1 with IL-1 β , LPS or anti-HMGB1 antibodies reduces the sensitivity of the conventional ELISA and causes false-negative results. Therefore, data from routine quantification of HMGB1 should be used with caution. Interestingly, anti-HMGB1 autoantibodies were

found in the sera not only of patients with rheumatic diseases and SLE but also in healthy subjects. Formation of complexes of these autoantibodies with HMGB1 may play an important role in limiting cytokine responses induced by HMGB1. Therefore, a new method to determine free and complex-bound HMGB1 has been proposed. The authors showed that the dissociation of preformed HMGB1/protein complexes by perchloric acid before ELISA allows the quantification of total HMGB1.

Modifications of HMGB1 by H₂S and SOD Mimics Could Regulate Its Activity: A Hypothesis on Potential Therapeutic Potential

The past decade has shown a lot of inconsistent effects of HMGB1 in the same pathological models. It seems that the effects of HMGB1 are tissue specific and often introduce additional contradictions. Despite the fact that HMGB1 acts as an early mediator of inflammation and organ damage in ischemia–reperfusion injury of the heart [with HMGB1 levels being already elevated 30 min after hypoxia *in vitro* and in ischemic injury of the heart *in vivo* (2)], some studies demonstrated beneficial effects of HMGB1 on myocardial regeneration after infarction. This effect occurs *via* enhanced cardiac C-Kit+ cell proliferation and differentiation (44). Further, modulation of the local inflammation in the chronically failing postinfarction myocardium has been shown to proceed particularly *via* lowered accumulation of dendritic cells. This resulted in attenuated fibrosis and cardiomyocyte hypertrophy, thereby improving global cardiac function (69). In addition, monoclonal anti-HMGB1 antibodies increased cell death and ROS generation in retinal ischemia–reperfusion (93).

However, when taking into consideration all the experimental results as of yet, it is important to notice that some of them dealt with the effects of endogenously released HMGB1 under specific stress situations (like ischemia–reperfusion), while others used externally added HMGB1 to modulate the existing pathological states. This may represent a major source for, sometimes, opposite effects of HMGB1 and may offer an idea for potential future therapeutic approaches. As mentioned above, HMGB1 is a redox-sensitive protein, and the redox status of its cysteine residues is affected by the pro-oxidative environment induced by ROS/RNS. This subsequently modulates its pathophysiological signals (84). Several chemical modifications could be envisioned that affect the activity of HMGB1 (Fig. 8).

Under the conditions of mild oxidative stress, Cys23 and 45 would probably be in form of a disulfide bridge, that is, active form, so it is the modification of Cys106 that determines the

FIG. 8. Hypothetical depiction of the possible roles of hydrogen sulfide (H₂S) and superoxide dismutase mimics (SODm) on structural modifications of HMGB1. The active form of HMGB1 (semioxidized), where Cys106 is reduced while Cys23 and 45 form disulfides, can be further modified depending on the extent of oxidative stress to form oxidized HMGB1, where Cys106 is in the form of sulfenic (RSOH), sulfinic (RSO₂H), or sulfonic (RSO₃H) acid. Only sulfenic acid can be reversed. Small amounts of H₂S could react with it to form sulfhydrylated HMGB1, while in the excess of H₂S, further breakage of the Cys23–45 disulfide bond can be achieved. On the other side, by reacting with NO, SODm dismutate it to nitrosonium (NO⁺) and nitroxyl (NO⁻/HNO) and induce formation of S-nitrosylated HMGB1 or HMGB1 dimers. The extent to which this modifications influence the HMGB1 remains to be elucidated.

direction of HMGB1 action. Under such conditions NO or its oxidized form (N_2O_3) could cause S-nitrosylation of Cys106. The magnitude of this modification has never been tested. It is tempting to speculate that the S-nitrosylation of Cys106 could be one way to affect the activity of HMGB1 or at least to modulate its mobility from the nucleus to the cytoplasm in an early stage of oxidative/nitrosative stress. At the site of inflammation, however, where more powerful oxidants are present (H_2O_2 and peroxynitrite among them), Cys106 would get oxidized to sulfenic (RSOH), sulfinic (RSO_2H), or sulfonic acid (RSO_3H), with only the first modification being readily reversible. Additionally, formation of dimers where two HMGB1 molecules are bridged *via* Cys106 could be envisioned as well. H_2S , besides scavenging peroxynitrite, can react as a reducing agent with both S-nitrosated HMGB1 and oxidized (RSOH) HMGB1. This would lead to the formation of S-sulphydrylated proteins, whose activity remains to be established. Further, H_2S can reduce disulfides between Cys23 and 45, forming the fully reduced and thus inflammatory inactive HMGB1. This could account for some of the beneficial effects of H_2S observed on the course of inflammation and can explain the increased production of H_2S observed under such conditions, where the latter would represent a mechanism of natural defense.

The role of SOD mimics would strongly depend on the class of the complexes, but it seems that all of them could be perfectly capable to reduce NO and to form HNO, as we discussed recently (33). HNO reacts with thiols to induce formation of sulphenylamine, which can further oxidize to form sulfinic or/and sulfonic acids. Alternatively, they can react with other free thiols to form disulfides. The use of SOD mimics would not only remove superoxide and/or NO, preventing thus peroxynitrite, but could also modulate HMGB1, leading to its either aggregation or oxidation. This would then represent an additional protective mechanism through which SOD mimics prevent necrotic cell death during inflammation and/or its further propagation due to inhibition of HMGB1.

As tempting as they look like, these hypotheses remain to be evaluated and deeper insight provided into actual modifications of HMGB1 that could occur at various physiological and pathophysiological situations. We hope that raising this as possibilities stimulates further research on these topics, as understanding of the magnitude by which these modifications could affect HMGB1 activity will lead to the design of small-molecule therapeutics for application at various pathological states.

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Abbreviations Used

ABC = ATP-binding cassette

Cys = cysteine

DAMP = death-associated molecular pattern

H₂S = hydrogen sulfide

HMGB1 = high-mobility group box protein 1

iNOS = inducible nitric oxide synthase

LPS = lipopolysaccharide

NADPH oxidase = nicotinamide adenine dinucleotide
phosphate oxidase

NF = nuclear factor

NO = nitric oxide

PARP-1 = poly[ADP-ribose] polymerase 1

RAGE = receptor for advanced glycation
end products

RNS = reactive nitrogen species

ROS = reactive oxygen species

SLE = systemic lupus erythematosus

SOD = superoxide dismutase

TLR = Toll-like receptor