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COMMENT

HMGB1: meeting the need for new tools in the box

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INTRODUCTION

Oral mucositis (OM) is a severe side effect of cancer therapy with no effective treatment. In a recent Mucosal Immunology paper, Im et al. showed that the investigational compound NecroX-7 was able to mitigate OM in murine models. It did so by limiting epithelial cell death and production of high mobility group box-1 (HMGB1) protein. HMGB1 has been implicated in the pathophysiology of a number of different diseases. However, there are many unresolved questions about how HMGB1 contributes to or prevents disease, making it critical to carefully evaluate both concentration and cellular context.

UNRESOLVED QUESTIONS REGARDING HMGB1

Among the most perplexing questions surrounding HMGB1 is how it can both activate and prevent cell death. The key to this apparent dichotomy lies, at least in part, in the location-dependent effects of this protein.[1] HMGB1 is found in the nucleus, cytosol, and extracellular space and is actively trafficked among these compartments. In each of these locations it has distinct functions that are also dependent upon post-translational modifications and its oxidative state. Although the forms and cellular localization of HMGB1 have been characterized in a number of different disease states, it is still unclear how to target HMGB1 therapeutically and which forms and sites should be the targets in specific diseases.

HMGB1 has been most extensively characterized as an extracellular, pro-inflammatory, pro-apoptotic molecule.[2] In fact, it was the first protein thought to fulfill the criteria described by Polly Matzinger for a damage-associated molecular pattern molecule or DAMP. HMGB1 can be released from cells through cell death or active secretion. Once it is in the extracellular space, it activates a number of pro-inflammatory receptors on target cells, often leading to cell death. The majority of the foundational studies identifying this function utilized methods such as pharmacologic inhibition, recombinant HMGB1, anti-HMGB1 antibody, or transformed cell lines. These methods were necessitated by the fact that the global HMGB1 knockout mouse dies within 24 h of birth.[3] However, they do provide some challenges to data interpretation. First, most pharmacologic inhibitors are not specific to HMGB1 so their effects could be direct or indirect through effects on upstream activators of HMGB1 expression or function. Second, the functions of recombinant HMGB1 appear to be at least somewhat dependent upon the cellular production system, since protein produced in *E. coli* is more pro-inflammatory than protein produced in mammalian cells.[4] Third, anti-HMGB1 antibody mitigates inflammation and tissue damage in a number of different rodent disease models, but its precise mechanism of action is still unknown. It could either inactivate functions of HMGB1 or limit the protein to a subset of functions by making specific sites more or less accessible. Finally, although transformed cell lines have been critical for mechanistic studies of cellular functions, their physiology differs from that of primary cells in a number of important ways. This means that findings in these systems may not be directly translatable to primary cells (Table 1).

The majority of the evidence supporting anti-apoptotic roles for HMGB1 has been derived from murine models. At least three independent mouse lines containing floxed HMGB1 genes have been generated. Each of these lines has now been used in a number of different conditional HMGB1 deficiency contexts, including different cell types and disease models. The initial findings from studies using these mice showed that inflammatory disease phenotypes were consistently exacerbated by loss of HMGB1 expression in relevant cell types. This was surprising in light of the well-accepted pro-inflammatory nature of HMGB1. Most strikingly, mice lacking HMGB1 in macrophages were more susceptible to lipopolysaccharide (LPS)-induced endotoxemia, without a change in the amount of circulating HMGB1.[5] This seemed at odds with the prevailing paradigm that HMGB1 is essential for inflammation in response to microbial ligands, including LPS. Considered together, findings from the initial murine studies supported a non-inflammatory, pro-survival role of HMGB1. This sparked a renewed interest in its intracellular functions, especially its role in the process of autophagy. There was also a group of murine models, including alcoholic liver disease and acetaminophen toxicity, in which loss of HMGB1 improved disease phenotypes.[6] These findings are perhaps more in keeping with the previously described extracellular role of HMGB1 or suggest that intracellular HMGB1 may be detrimental in some contexts. Although in vivo animal studies are generally considered the most physiologically relevant disease models there are still challenges to data interpretation. In particular, conditional HMGB1 knockout leads to both intracellular deficiency and decreased release of HMGB1 from deficient cells. Furthermore, HMGB1 can still be produced and released from other cell types. This is important since this protein may have unique roles in different cell types or cellular contexts.

ANSWERING THE QUESTIONS SURROUNDING HMGB1 FUNCTIONS

Sorting out this confusion to allow therapeutic targeting of HMGB1 requires careful dissection of the site and form of HMGB1 responsible for cellular and disease phenotypes. The recent work by Im et al. in Mucosal Immunology represents an important step in this path.[7] These authors describe the first use of endogenous HMGB1 overexpression in the gastrointestinal tract and join the small number of publications describing endogenous

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Table. 1. Experimental tools available to study the function of HMGB1 and their considerations for use

Experimental Tool	Considerations
Global HMGB1 knockout mice	Mice die 24 h after birth
Conditional HMGB1 knockout mice	Impacts expression in more than one cellular compartment (nuclear, cytosolic, and/or extracellular)
Pharmacologic inhibitors of HMGB1	May not be specific to HMGB1
Recombinant HMGB1 proteins	Cellular source influences function
HMGB1 blocking antibody	Precise mechanism of action is unknown
In vivo overexpression systems	Impacts expression in more than one cellular compartment (nuclear, cytosolic, and/or extracellular)

overexpression of HMGB1 in vivo. In this work, they used intravenous delivery of an adeno-associated viral vector (AAV9-CMV-HMGB1-Luc) to manipulate HMGB1 expression. In vivo bioluminescence showed that HMGB1-Luciferase was primarily localized to the tongue, liver, and gastrointestinal tract with lesser expression in the spleen, heart, and kidney. The authors reported no pathology in the untreated mice. However, when the mice were treated with 5-fluorouracil (5-FU), HMGB1 overexpressing mice experienced more severe OM with larger tongue ulcers, a thinner epithelial layer, and increased epithelial cell death versus controls. Consistent with the increased cell death and mucosal barrier failure, HMGB1 overexpressing mice also had increased mRNA expression of inflammatory cytokines versus controls. The authors utilized this model of HMGB1 overexpression to support experiments showing that the mitochondrial antioxidant NecroX-7 mitigated OM by decreasing HMGB1 production and epithelial cell death. However, they did not determine whether NecroX-7 could mitigate OM when HMGB1 was overexpressed.

Oral mucositis is ulceration of the tongue and oral mucosa that occurs as a complication in the majority of cancer patients receiving chemotherapy or radiation.[8] It is one of the most common and important side effects of treatment with the chemotherapeutic agent 5-fluorouracil (5-FU). Since there are no consistently effective treatments for OM, it often leads to delay or discontinuation of treatment for the underlying cancer.[8] This makes it important to understand and prevent this complication. Though HMGB1 levels and cellular distribution have not yet been characterized in patients with OM, there are a number of studies that have implicated HMGB1 in this disease. A previous study showed that 5-FU treatment leads to HMGB1 release from colon carcinoma cells.[9] Literature has also shown that HMGB1 knockout decreases death of mouse embryonic fibroblasts exposed to 5-FU and that inhibition of autophagy with 3-methyladenine significantly impairs HMGB1 release from cells after 5-FU treatment.[9,10] Lastly, treatment of cells with anthocyanins, compounds with a similar action to NecroX-7, decreases HMGB1 release from cells and OM in mice treated with 5-FU.[11] Taken together with the report from Im et al., a model emerges in which OM results from epithelial cell death and release of pro-inflammatory proteins, such as HMGB1, from the dying cells.

REMAINING QUESTIONS AND POTENTIAL FOR THE FUTURE

Like any good work, the study by Im et al. raises additional questions that present opportunities for new discoveries. This study did not specifically examine the role of HMGB1 in different cellular compartments, although their HMGB1 overexpression model would be expected to increase both intracellular and extracellular HMGB1 expression. Since 5-FU causes release of HMGB1 into the extracellular compartment, extracellular functions are likely most important in this model. However, it doesn't completely rule out intracellular roles for HMGB1. In fact, translocation could lead to loss of intracellular and gain of extracellular functions for HMGB1 in this model. Our previous work identified a role for HMGB1 in preservation of autophagy and prevention of apoptosis in intestinal epithelial cells; which would be lost if HMGB1 moves from the cytosol to the extracellular space.[12] In light of these factors, a careful characterization of the concentrations of HMGB1 in the epithelial cell nucleus, cytosol, and extracellular space using their model would be very interesting.

This model would also allow examination of the mechanisms through which 5-FU causes release of HMGB1 from cells, potential therapeutic targets for OM. These mechanisms could be related to processes that lead to active secretion of this protein or passive release when cells die. NecroX-7 is an antioxidant so it may directly act on HMGB1 or HMGB1-specific pathways or it may act on upstream pathways to prevent cell death and passive release of this protein. Epithelial cell death is a major component of the pathophysiology of OM and is almost certainly an important factor in changes in HMGB1 localization in this model. Therefore, examining the mechanisms behind cell death may be a clue to understanding when HMGB1 is protective and when it contributes to cell death. Studies like the one reported by Im et al., in which HMGB1 expression is manipulated using endogenous methods in physiologic models, offer a wealth of opportunity to better understand not only this protein, but also the overarching principles of cell death and how they contribute to disease.

AUTHOR CONTRIBUTIONS

AMCO and JSM together conceived and wrote this commentary.

ADDITIONAL INFORMATION

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