

Cancer Cell Secretion of the DAMP Protein HMGB1 Supports Progression in Malignant Mesothelioma

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Abstract

Human malignant mesothelioma is an aggressive and highly lethal cancer that is believed to be caused by chronic exposure to asbestos and erionite. Prognosis for this cancer is generally poor because of late-stage diagnosis and resistance to current conventional therapies. The damage-associated molecular pattern protein HMGB1 has been implicated previously in transformation of mesothelial cells. Here we show that HMGB1 establishes an autocrine circuit in malignant mesothelioma cells that influences their proliferation and survival. Malignant mesothelioma cells strongly expressed HMGB1 and secreted it at high levels *in vitro*. Accordingly, HMGB1 levels in malignant mesothelioma patient sera were higher than that found in healthy individuals. The motility, survival, and anchorage-independent growth of HMGB1-secreting malignant mesothelioma cells was inhibited *in vitro* by treatment with monoclonal antibodies directed against HMGB1 or against the receptor for advanced glycation end products, a putative HMGB1 receptor. HMGB1 inhibition *in vivo* reduced the growth of malignant mesothelioma xenografts in severe-combined immunodeficient mice and extended host survival. Taken together, our findings indicate that malignant mesothelioma cells rely on HMGB1, and they offer a preclinical proof-of-principle that antibody-mediated ablation of HMGB1 is sufficient to elicit therapeutic activity, suggesting a novel therapeutic approach for malignant mesothelioma treatment. *Cancer Res*; 72(13): 3290–301. ©2012 AACR.

Introduction

Human malignant mesothelioma arises from the neoplastic transformation of mesothelial cells lining the pleural, peritoneal, and pericardial cavities. Malignant mesothelioma has been linked to occupational and environmental exposure to asbestos, causing more than 100,000 deaths per year worldwide (1). Moreover, in rapidly industrializing countries, such as India and China, where the use of asbestos is unrestricted, the incidence of malignant mesothelioma is expected to rise dramatically (2). Erionite, a natural mineral fiber that can be dispersed in the environment by human activities also causes malignant mesothelioma (3). We have recently discovered extensive erionite exposure in the United States (4). It has been estimated that more than 25 million people have been exposed to asbestos in the United States, whereas the number of those exposed to erionite is still unknown (1, 4).

Malignant mesothelioma is a very aggressive cancer, usually diagnosed at late stages when it is refractory to most therapeutic modalities, leading to poor prognosis with a patients' median survival of 8 to 12 months from diagnosis. Malignant mesothelioma is considerably resistant to all current treatments, and survival may only be extended by about 11 weeks in patients treated with Cisplatin/Alimta as the standard of care (5–7). However, in the 5% of patients diagnosed at an early stage (Stage Ia), survival of 5 or more years is not uncommon (5–7). Therefore, the development of new biomarkers for early detection and of novel targets for preventive and therapeutic approaches to malignant mesothelioma are most needed. Moreover, recently we discovered a novel cancer syndrome caused by *BAP1* germline mutations, characterized by the development of uveal melanoma and mesothelioma and possibly other cancers (8). When individuals with *BAP1* mutations are exposed to asbestos or erionite, mesothelioma predominates. Thus, it has become possible to identify within asbestos and erionite exposed cohorts those individuals at the highest risk of mesothelioma for early diagnosis.

We recently showed that asbestos- and erionite-exposed primary human mesothelial cells release High Mobility Group Box 1 protein (HMGB1), which plays a critical role in the carcinogenesis of these mineral fibers (4, 9). HMGB1 is a damage-associated molecular pattern (DAMP) and a key mediator of inflammation (10). Although HMGB1 is a nuclear protein, it is detected in the cytoplasm of cells undergoing necrosis and in some cell types that can actively secrete it, such as macrophages. Once in the extracellular space, HMGB1 binds

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to the receptor for advanced glycation endproducts (RAGE; ref. 11) and to the Toll-like receptors (TLRs 2 and 4; ref. 12) starting the inflammatory process (13–17). HMGB1 induces the secretion of TNF- α by macrophages and activation of NF- κ B, a key regulator of oncogenesis (9). Activation of NF- κ B promotes cell proliferation and inhibits cell death, leading to enhanced survival of human mesothelial cells that have accumulated DNA alterations following asbestos exposure, thus facilitating their malignant transformation (18).

Malignant mesothelioma biopsies often show a marked inflammatory infiltrate that contains a large number of tumor-associated macrophages. Here we show that HMGB1 is highly expressed and secreted by malignant mesothelioma cells, establishing an autocrine circuit. Consistently, mesothelioma patients have elevated HMGB1 serum levels, suggesting that HMGB1 may be a novel biomarker for malignant mesothelioma. In addition, inhibition of HMGB1 impaired the motility, survival, and anchorage-independent growth of HMGB1-secreting mesothelioma cells *in vitro*. Finally, a monoclonal antibody (mAb) against HMGB1 reduced tumor growth in xenografted severe-combined immunodeficient (SCID) mice, extending their survival.

Our data indicate that the sustained release of HMGB1 by malignant mesothelioma cells, along with its secretion by surrounding inflammatory cells, supports the malignant phenotype of mesothelioma. These findings provide the rationale for inhibiting HMGB1 as a novel molecular targeted therapy of malignant mesothelioma.

Materials and Methods

Cell cultures

Primary human mesothelial cells were obtained from pleural effusions of 8 different patients, pathologically diagnosed free of malignancy. Human mesothelial cells were characterized and cultured as previously described (19). Malignant mesothelioma cell lines were established from surgically resected human malignant mesothelioma specimens. REN cells were provided by Dr. Steven Albelda (University of Pennsylvania, Philadelphia, PA), whereas all other cell lines used in this study were provided by Dr. Harvey I. Pass (NYU). REN/luc luciferase were generated as previously described (20).

Reagents and materials

Full-length, lipopolysaccharide (LPS)-free purified HMGB1 and BoxA were obtained from HMGBiotech, the neutralizing monoclonal anti-HMGB1 (DPH1.1) was from Dia.Pro Diagnostics; the neutralizing monoclonal anti-RAGE (Clone #176902) was from R&D Systems, normal mouse IgG was from Sigma-Aldrich. Crocidolite asbestos was obtained from the Union Internationale Contre le Cancer (Switzerland) and processed as previously described (9, 19).

Immunohistochemistry

Immunohistochemistry was carried out on human malignant mesothelioma and normal pleura paraffin-embedded tissues with rabbit polyclonal anti-HMGB1 (Abcam). Goat anti-rabbit secondary antibody and Vectastain Elite ABC Kit

(Rabbit IgG; Vector Labs) were used according to the manufacturer's instructions. HMGB1 immunostaining was analyzed blindly by 2 board certified pathologists (A.P. and M.C.).

Immunocytochemistry

Immunocytochemistry on malignant mesothelioma and primary mesothelial cells was carried out using the Vectastain ABC Kit (Vector Labs) according to the manufacturer's instructions. Mouse monoclonal anti-HMGB1 (Abcam) was used for the detection of intracellular HMGB1 protein.

HMGB1 ELISA

The human HMGB1 ELISA Kit (IBL International) was used to measure the levels of HMGB1 in patients' sera and in conditioned media of primary mesothelial cells and malignant mesothelioma cell lines. Samples were tested in duplicate. Sera were obtained from 20 untreated (prechemotherapy and presurgery) mesothelioma patients and 20 age- and gender-matched healthy individuals. All participants provided informed consent, and procedures and protocols were approved by the Institutional Review Board. For the detection of extracellular HMGB1 released by malignant mesothelioma cell lines and human primary mesothelial cells, 2×10^6 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 1% FBS for 24 hours. The culture media were then collected and concentrated by ultrafiltration using Amicon Ultra Centrifugal Filters (Millipore), and 10- μ L aliquots were assayed in duplicate by ELISA. All culture media were collected under identical condition.

Quantitative real-time PCR

Total RNA from malignant mesothelioma and human primary mesothelial cells was isolated using RNeasy kit (Qiagen) and treated with RNase-free DNase. The following primers from Qiagen were used: Hs_AGER_1_SG (QT00000119), Hs_HMGB1_1_SG (QT01002190), Hs_TLR4_2_SG (QT01670123), and Hs_TLR2_1_SG (200) (QT00236131) to amplify the respective cDNAs as previously described (18).

Western blotting

Human primary mesothelial cells and malignant mesothelioma cells were lysed and the cytoplasmic and nuclear fractions separated using the protein extraction kit from Active Motif, according to the manufacturer's instructions. A total of 50 μ g of protein lysates were used for Western blotting carried out as previously described (18), using mouse monoclonal anti-HMGB1, rabbit polyclonal anti-RAGE, mouse monoclonal anti-TLR2, and goat polyclonal anti-TLR4 (Abcam). Anti- α -Tubulin (Calbiochem) and anti-Lamin B (Abcam) were used as loading controls for the cytoplasmic and nuclear fractions, respectively.

Viability and cytotoxicity assays

Malignant mesothelioma cells (1×10^4 per well) were incubated for 24 hours in DMEM with 1% FBS containing one of the HMGB1 antagonists: BoxA (100 ng/mL), anti-HMGB1 (1.0 μ g/mL), or anti-RAGE (1.7 μ g/mL). Mouse IgG (1.7 μ g/mL)

was used as control. The CellTiter 96 Aqueous Cell Proliferation Assay-MTS (Promega) was used to evaluate cell viability, and the lactate dehydrogenase (LDH) cytotoxicity detection kit (Roche) was used to evaluate cytotoxicity.

Migration and invasion assays

The *in vitro* cell migration and invasion assays were carried out using Costar Transwell permeable polycarbonate supports (8.0- μm pores) in 24-well plates (Corning Inc.). For the migration assays, 1×10^5 malignant mesothelioma cells in serum-free DMEM were used. For the invasion assays, 2×10^5 malignant mesothelioma cells in serum-free DMEM were seeded in the upper compartment coated with Matrigel. The lower compartment contained serum-free DMEM (negative control) or DMEM plus 10% FBS (positive control), purified recombinant HMGB1 (100 ng/mL), or concentrated medium (200 μL) from REN or Mill cells.

Wound healing assay

Malignant mesothelioma cells were seeded in 6-well plates and grown to 80% to 90% confluence in DMEM plus 1% FBS. One hour before wounding, the cells were treated with either BoxA (100 ng/mL), anti-HMGB1 (1.0 $\mu\text{g}/\text{mL}$), anti-RAGE (1.7 $\mu\text{g}/\text{mL}$), or IgG control (1.7 $\mu\text{g}/\text{mL}$). The cell monolayer was wounded with a P200 pipette tip, and wound closure was observed after 48 hours.

Soft agar assay

Anchorage-independent cell proliferation (REN 4×10^3 cells) was determined by the soft agar assay. After 23 days of culture, the number and size of the colonies formed in each treatment [BoxA (100 ng/mL), anti-HMGB1 (1.0 $\mu\text{g}/\text{mL}$), anti-RAGE (1.7 $\mu\text{g}/\text{mL}$), or irrelevant IgG control (1.7 $\mu\text{g}/\text{mL}$)] were evaluated.

SCID human malignant mesothelioma xenografts

Female SCID mice aged 6 to 8 weeks (Jackson Laboratories) were housed and handled under aseptic conditions, in accordance with our institution's Institutional Animal Care and Use Committee (IACUC) guidelines. Twenty-one SCID mice were injected intraperitoneally with 5×10^5 REN/luc cells suspended in 500 μL of PBS, as described (20, 21). Xenografts were visualized by luminescence after D-luciferin injection (150 mg/kg) using the In Vivo Imaging System (IVIS, Xenogen Corp.), with regions of interest quantified as total photon counts by Living Image software (Xenogen Corp.). Four days were required for the formation of detectable tumor nodules by IVIS imaging. Mice were then weighed and randomly assigned to control (IgG isotype control or PBS) and treatment (anti-HMGB1 mAb) groups of 7 animals each. The "treatment" group received 200 μg i.p. every 2 days for the first week, then every week until day 38, for a total of 1.8 mg/mouse of anti-HMGB1 mAb. Control groups received either intraperitoneal injections of matched isotype control IgG (200 $\mu\text{g}/\text{injection}$) or PBS with the same schedule as the anti-HMGB1-treated group. Tumor dimension was measured every 7th day as average radiance (photons/s/cm²/sr). The majority of the animals died spontaneously, except one mouse in the anti-HMGB1 group died

accidentally from a misplaced injection and was not counted toward the survival analysis; 2 mice, one each from vehicle and anti-HMGB1 groups, were euthanized and necropsied when tumor development caused severe ascites limiting the animal's mobility, according to IACUC regulations.

Statistical analysis

Where not otherwise indicated, statistical significance between 2 groups of interest was evaluated by unpaired Student *t* test. Differences were considered significant at $P < 0.05$. Differences in the HMGB1 levels in human sera were analyzed by paired *t* test. The association between HMGB1 and RAGE mRNA levels in malignant mesothelioma cell lines and the association between tumor stage and HMGB1 cytoplasmic staining in malignant mesothelioma tissues were assessed by calculating the Pearson correlation coefficient (*r*). For the SCID malignant mesothelioma xenografts experiment, a 2-way ANOVA assessed the effects of treatment, time, and the treatment by time interaction on weight. Bonferroni-corrected posttests compared HMGB1 mAb to the control groups (PBS or IgG controls) at each time point. Differences of survival across groups were assessed by fitting a parametric model to the survival time data; a Weibull distribution was assumed; the LIFEREG procedure in SAS 9.2 carried out the analysis.

Results

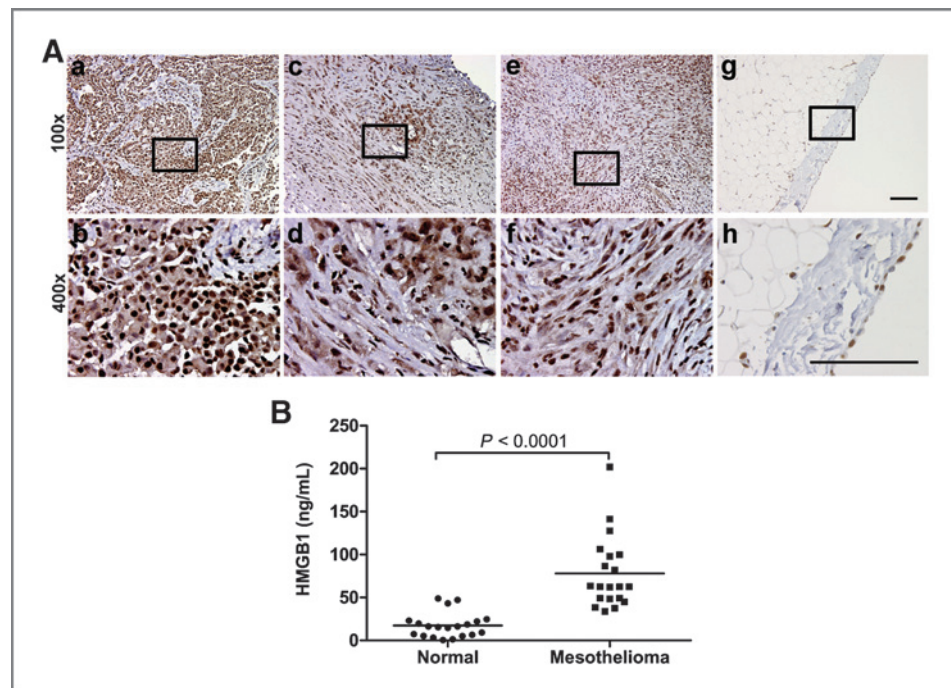
HMGB1 inhibitors hinder asbestos-induced human mesothelial cells transformation

In vivo, macrophages are recruited to the sites of asbestos deposition (22) where they are known to release proinflammatory cytokines into the microenvironment (9, 18). To mimic the cross-talk between human mesothelial cells and macrophages, we developed a coculture system in which human mesothelial cells form tridimensional foci about 1 to 2 months after asbestos exposure (4). Using this assay, we tested 2 different HMGB1 inhibitors, BoxA (23) and an anti-HMGB1-neutralizing mAb (24). The number of foci (mean \pm SEM) formed in the HM-macrophages cocultures treated with either BoxA (53.5 ± 6.4) or HMGB1 mAb (70.0 ± 9.9) was significantly lower than in the untreated cocultures (136.5 ± 7.8 ; $P < 0.05$, Supplementary Fig. S1). Moreover, a 2-week delay in the initial development of foci was observed in HMGB1-neutralized cocultures. These results showed that these HMGB1 inhibitors interfere with asbestos-induced human mesothelial cells transformation.

HMGB1 is highly expressed in malignant mesothelioma tissues and sera of malignant mesothelioma patients

Because malignant mesothelioma biopsies often show a marked inflammatory infiltrate, we tested whether HMGB1 might be also involved in maintaining chronic inflammation in the malignant mesothelioma microenvironment after the establishment of cell transformation. We analyzed HMGB1 in 31 mesothelioma biopsies representing all 3 main histologic subtypes of mesothelioma (21 epithelioid, 6 biphasic and 4 sarcomatoid). All the mesothelioma biopsies showed uniform strong nuclear staining (Fig. 1A). Most

Figure 1. HMGB1 is highly expressed in malignant mesothelioma tissues and sera of mesothelioma patients. **A**, strong expression of HMGB1 was detected in the nuclei of 31 of 31 mesothelioma biopsies representing all 3 main histologic subtypes of malignant mesothelioma: epithelial (a, b), biphasic (c, d), and sarcomatoid (e, f). In 26 of 31 mesothelioma biopsies HMGB1 is detected in both nucleus and cytoplasm. In the single-cell mesothelial layer of normal pleura (g, h), HMGB1 is only detected in nucleus. Rectangles in $\times 100$ magnification pictures (top) indicate the area shown in $\times 400$ magnification (bottom). Scale bar, 100 μm . **B**, HMGB1 levels in sera of 20 mesothelioma patients are significantly higher ($P < 0.0001$) than in 20 healthy individuals. Bars show mean of HMGB1 levels.



mesothelioma specimens also showed a variable degree of cytoplasmic staining (epithelioid: 17 of 21; biphasic: 5 of 6; sarcomatoid: 4 of 4; Fig. 1A; Table 1). In those specimens that were scored negative, there were focal areas of cytoplasmic positivity, usually corresponding to clusters of invading tumor cells. Moreover, statistical significance ($r = 0.61$, $P = 0.002$) was found in the correlation between tumor stage and HMGB1 cytoplasmic staining in the tissues. The higher tumor stage was associated with stronger HMGB1 staining; however, further research using a larger sample size may be needed to validate this correlation. In normal pleura, HMGB1 staining was fainter and was localized only in the nucleus (Fig. 1A).

Because cytoplasmic HMGB1 is usually associated with HMGB1 secretion or release, these data suggested that HMGB1 could be secreted or released into the extracellular space, making its way into the patient's blood. We tested HMGB1 levels in serum samples from 20 malignant mesothelioma patients and 20 age- and gender-matched healthy individuals. HMGB1 concentration (mean \pm SEM) in mesothelioma patients' sera was 77.9 ± 9.4 ng/mL, significantly higher than that in sera from healthy controls (17.5 ± 3.2 ng/mL, $P < 0.0001$; Fig. 1B).

The high levels of HMGB1 expression in the majority of mesothelioma biopsies tested, its cytoplasmic localization, as well as its high levels in sera of mesothelioma patients suggested that extracellular HMGB1 might be relevant to the biology of malignant mesothelioma cells.

HMGB1 and RAGE are upregulated in malignant mesothelioma cells

We investigated the expression of HMGB1 and its receptors RAGE, TLR2, and TLR4 in a panel of 6 malignant mesothelioma

cell lines and 6 distinct primary human mesothelial cell cultures. Quantitative reverse transcriptase PCR revealed that in 5 of 6 malignant mesothelioma cell lines, the amount of HMGB1 transcripts was significantly higher compared with primary mesothelial cells. A large degree of variability was observed: Ada and Mill cells expressed relatively low levels of HMGB1 transcripts, whereas in REN, Hmeso and Phi cells, the amount of HMGB1 transcripts exceeded that found in primary human mesothelial cells by 6, 10, and 20 times, respectively (Fig. 2A).

Cells with abundant HMGB1 transcripts (Phi, REN, and Hmeso) also had higher amounts of RAGE transcripts, whereas cells with low HMGB1 mRNA (Ada and Mill) also had low levels of RAGE mRNA (Fig. 2B). We found that the correlation between HMGB1 and RAGE mRNA levels in the 5 different malignant mesothelioma cell lines tested was statistically significant ($r = 0.93$, $P = 0.022$; Fig. 2F). TLR2 and TLR4 transcripts were also higher in malignant mesothelioma cells than in primary mesothelial cells, although their overall levels were much lower than for RAGE (Supplementary Fig. S2A).

The subcellular compartmentalization of HMGB1 protein was determined by cell fractionation and Western blotting. In human primary mesothelial cells, HMGB1 was almost exclusively detected in the nuclear fraction; instead, malignant mesothelioma cells contained high amounts of HMGB1 in both the nucleus and the cytoplasm (Fig. 2C). These results were confirmed by immunostaining: Malignant mesothelioma cells had both nuclear and cytoplasmic HMGB1-positive staining, whereas primary mesothelial cells had exclusively nuclear staining (Fig. 2E), a result that was in accordance with the findings in malignant mesothelioma biopsies (Fig. 1A).

Table 1. Stage and cytoplasmic HMGB1 expression of malignant mesothelioma cases

Specimen ID	Subtype	Stage	Score
SP-001	E	III	3+
SP-009	E	II	0
SP-011	E	II	1+
SP-012	E	III	2+
SP-013	B	III	1+
SP-014	E	III	1+
SP-015	E	IV	1+
SP-016	E	Unknown	1+
SP-018	E	III	1+
SP-019	S	III	1+
SP-020	E	Unknown	1+
SP-021	E	I	0
SP-022	E	Unknown	1+
SP-023A	B	Unknown	1+
SP-023B	B	Unknown	1+
SP-024	E	III	1+
SP-025	E	III	1+
SP-026	E	Unknown	1+
SP-027	B	IV	3+
SP-028	S	III	3+
SP-029	S	IV	2+
SP-030	E	III	1+
SP-031	E	III	0
SP-032	B	I	0
SP-033	E	Unknown	1+
SP-034	E	II or III	1+
SP-035	B	III	2+
SP-036	S	IV	2+
SP-037	E	IV	1+
SP-038	E	IV	2+
SP-039	E	II	0

NOTE: Correlation between tumor stage and cytoplasmic HMGB1: $r = 0.61$; $P = 0.002$.

Abbreviations: E, epithelioid; B, biphasic; S, sarcomatoid.

Consistent with the results on RAGE, TLR2, and TLR4 transcripts, the corresponding proteins were expressed at higher levels in malignant mesothelioma cells (Fig. 2D and Supplementary Fig. S2B).

These results indicated that most malignant mesothelioma cell lines express high levels of both HMGB1 and its main receptors RAGE, TLR2, and TLR4, suggesting that HMGB1 signaling may influence the phenotype of malignant mesothelioma.

HMGB1 induces migration and proliferation of malignant mesothelioma cells

HMGB1 induces migration and proliferation in certain cell types (25, 26). Therefore, we tested its activity also on malignant mesothelioma cells. Both REN and Phi cells migrated

toward purified recombinant human HMGB1 (100 ng/mL), whereas the incubation with anti-RAGE mAb abrogated cell migration (Supplementary Fig. S3A and B). Recombinant HMGB1 also significantly increased the proliferation rate of malignant mesothelioma cells REN and Phi (Supplementary Fig. S3C). Downregulation of HMGB1 with 2 different gene-specific short hairpin RNA (shRNA) constructs significantly inhibited cell proliferation of REN cells compared with cells transfected with a scrambled control noneffective shRNA (Supplementary Fig. S3D).

These experiments indicated that HMGB1 is a chemotactic and mitogenic factor for malignant mesothelioma cells.

HMGB1 is an autocrine motility factor for malignant mesothelioma cells

We found that significantly higher levels of HMGB1 were present in the 24 hours conditioned media of 6 of 7 malignant mesothelioma cell lines, compared with human primary mesothelial cells (both primary mesothelial cells and malignant mesothelioma were at 80%–85% confluence). Consistently, Mill and human mesothelial cells, with low HMGB1 expression, released barely detectable amounts of HMGB1 (Fig. 3A).

To verify the possible occurrence of an autocrine loop, we collected and concentrated the conditioned medium from REN cells (high HMGB1 producers) and from Mill (low HMGB1 producers) and tested their chemoattractant activity. Concentrated conditioned media (CCM) from REN cells induced the migration of REN cells themselves (Fig. 3B) and Phi cells (Supplementary Fig. S4A). In both cell lines, cell motility was blocked by BoxA. Instead, CCM collected from Mill cells did not induce a chemotactic response in any of the 2 malignant mesothelioma cell lines tested (Fig. 3C and Supplementary Fig. S4B).

These results showed that HMGB1 secreted by malignant mesothelioma cells is biologically active and induces migration of the same cells in an autocrine fashion.

Malignant mesothelioma cells require HMGB1 for viability

Because recombinant HMGB1 enhances the growth rate of malignant mesothelioma cells, we tested whether the HMGB1 secreted by mesothelioma cells plays a role in their survival.

The viability of REN, Phi, and Mill cells was tested following HMGB1 inhibition. BoxA, anti-HMGB1, and anti-RAGE antibodies significantly reduced the viability of REN and Phi cells (Fig. 4A) but had only mild or no effects on Mill cells (Supplementary Fig. S5A).

Both anti-HMGB1 and anti-RAGE antibodies induced marked cytotoxicity in REN and Phi cells (Fig. 4B), whereas a mild cytotoxic effect was observed in Mill cells only when treated with anti-RAGE antibody (Supplementary Fig. S5B), compared with untreated controls ($P < 0.05$). In primary human mesothelial cells, HMGB1 inhibition did not cause any change in cell viability or proliferation or cytotoxicity (Supplementary Fig. S6A–C).

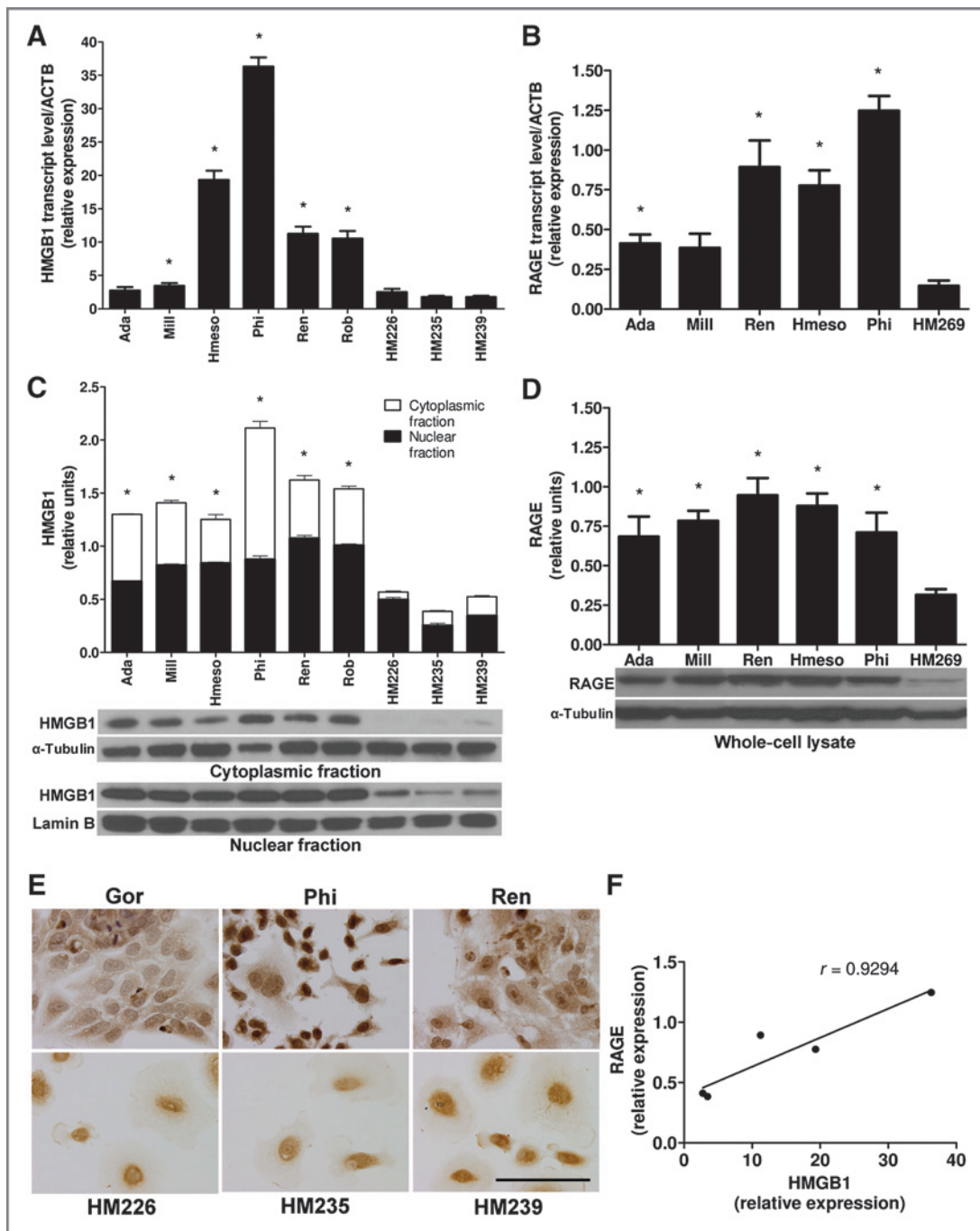


Figure 2. HMGB1 and RAGE expression are both upregulated in malignant mesothelioma cells. **A**, HMGB1 mRNA levels are higher in malignant mesothelioma than in human primary mesothelial cells (HM). Different primary mesothelial cell cultures have similar low HMGB1 levels (Ada, Mill, Hmeso, Phi, Ren, and Rob are malignant mesothelioma cell lines; HM226, HM235, and HM239 are 3 representative human mesothelial cells that are shown). *, $P < 0.05$; malignant mesothelioma versus human mesothelial cells. **B**, RAGE mRNA levels are higher in malignant mesothelioma than in human mesothelial cells. Experiments were conducted as in **A**. *, $P < 0.05$; malignant mesothelioma versus primary mesothelial cells. **C**, cell compartmentalization. Total HMGB1 protein levels are higher in malignant mesothelioma cells than in primary human mesothelial cells, and HMGB1 is localized in both nucleus and cytoplasm of malignant mesothelioma cells but mainly in the nucleus of primary mesothelial cells. α -Tubulin and Lamin B, loading controls for the cytoplasmic and nuclear fractions, respectively. HMGB1 relative densitometry units were calculated. *, $P < 0.05$; malignant mesothelioma cells versus primary mesothelial cells. **D**, Western blotting shows higher RAGE expression in malignant mesothelioma cells than in primary mesothelial cells. α -Tubulin, loading control. RAGE relative densitometry units were calculated. *, $P < 0.05$; malignant mesothelioma cells versus primary mesothelial cells. All the experiments were carried out 3 times; error bars represent SEM. **E**, immunocytochemistry. HMGB1 is detected in both nucleus and cytoplasm of malignant mesothelioma cells but mainly in the nucleus of primary human mesothelial cells (3 representative malignant mesothelioma cell lines and 3 representative primary mesothelial cells are shown). Original magnification, $\times 400$. Scale bar, 100 μ m. **F**, HMGB1 and RAGE transcript levels show significant positive correlation in 5 different malignant mesothelioma cell lines tested ($r = 0.93$, $P = 0.022$).

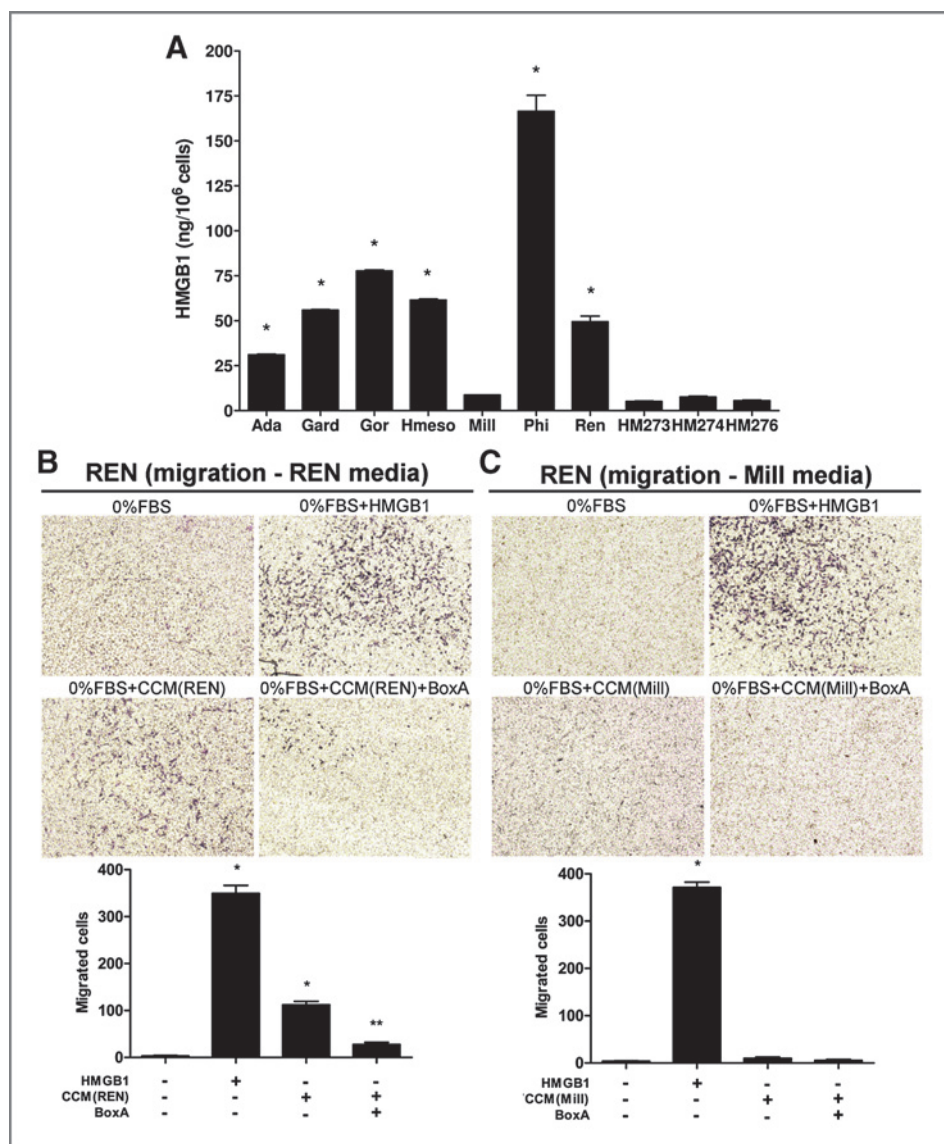


Figure 3. HMGB1 secreted by malignant mesothelioma cells is biologically active. A, malignant mesothelioma cells (Ada, Gard, Gor, Hmeso, Mill, Phi, Ren) release larger amounts of HMGB1 in the culture media than primary human mesothelial cells (HM273, HM274, HM276), as measured by ELISA. Culture media for the different cells were collected and concentrated under identical condition. Experiments were done in duplicate and carried out twice. *, $P < 0.05$; malignant mesothelioma cells versus primary mesothelial cells. B, CCM collected from REN cells induce the migration of REN cells themselves. Migration is blocked by BoxA. C, CCM collected from Mill cells did not induce a significant chemotactic response in REN cells. All CCM were collected under identical conditions. Migrated cells were counted using ImageJ software and represent mean values per field from at least 3 fields. Experiments were done in duplicate. *, $P < 0.05$; HMGB1 and CCM from REN/Mill versus 0% FBS. **, $P < 0.05$; CCM alone versus CCM plus BoxA. In all panels, error bars represent SEM.

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We further analyzed cell death induced by HMGB1 antagonists. Flow cytometry revealed that BoxA, anti-HMGB1, and anti-RAGE antibodies significantly induced apoptosis in REN cells (Supplementary Fig. S7A and B), but not in Mill cells (Supplementary Fig. S7C and D).

These results strongly suggested that malignant mesothelioma cells secreting high levels of HMGB1 are "addicted" to HMGB1 for their viability.

HMGB1 is required for malignant mesothelioma cell motility

All HMGB1 inhibitors significantly reduced wound closure of REN (Fig. 4C and D) and Phi (Fig. 4D) cells (high HMGB1 producers), but there was no effect on low HMGB1 producers Mill cells (Supplementary Fig. S5C and D). The results showed that HMGB1 is critical for the motility of malignant mesothelioma cells that secrete it.

HMGB1 inhibition disrupts invasiveness and anchorage-independent growth of malignant mesothelioma cells

We carried out a Matrigel invasion assay with REN (Fig. 5A) and Phi cells (Supplementary Fig. S8). Recombinant HMGB1 significantly enhanced the invasion of Matrigel by both cell lines, and the anti-RAGE mAb significantly reduced malignant mesothelioma cell invasion induced by HMGB1.

To verify anchorage-independent growth, we carried out soft agar assays. All HMGB1 inhibitors (BoxA, anti-HMGB1, and anti-RAGE) caused a significant decrease in REN anchorage-independent growth, as indicated by a marked reduction in the number (Fig. 5B) and size (Fig. 5C) of colonies.

Expression analysis, carried out with the Affymetrix HumanGene 1.0 ST array, revealed that stimulation with HMGB1 enhanced the transcription of multiple genes controlled by the activation of NF- κ B and downstream genes.

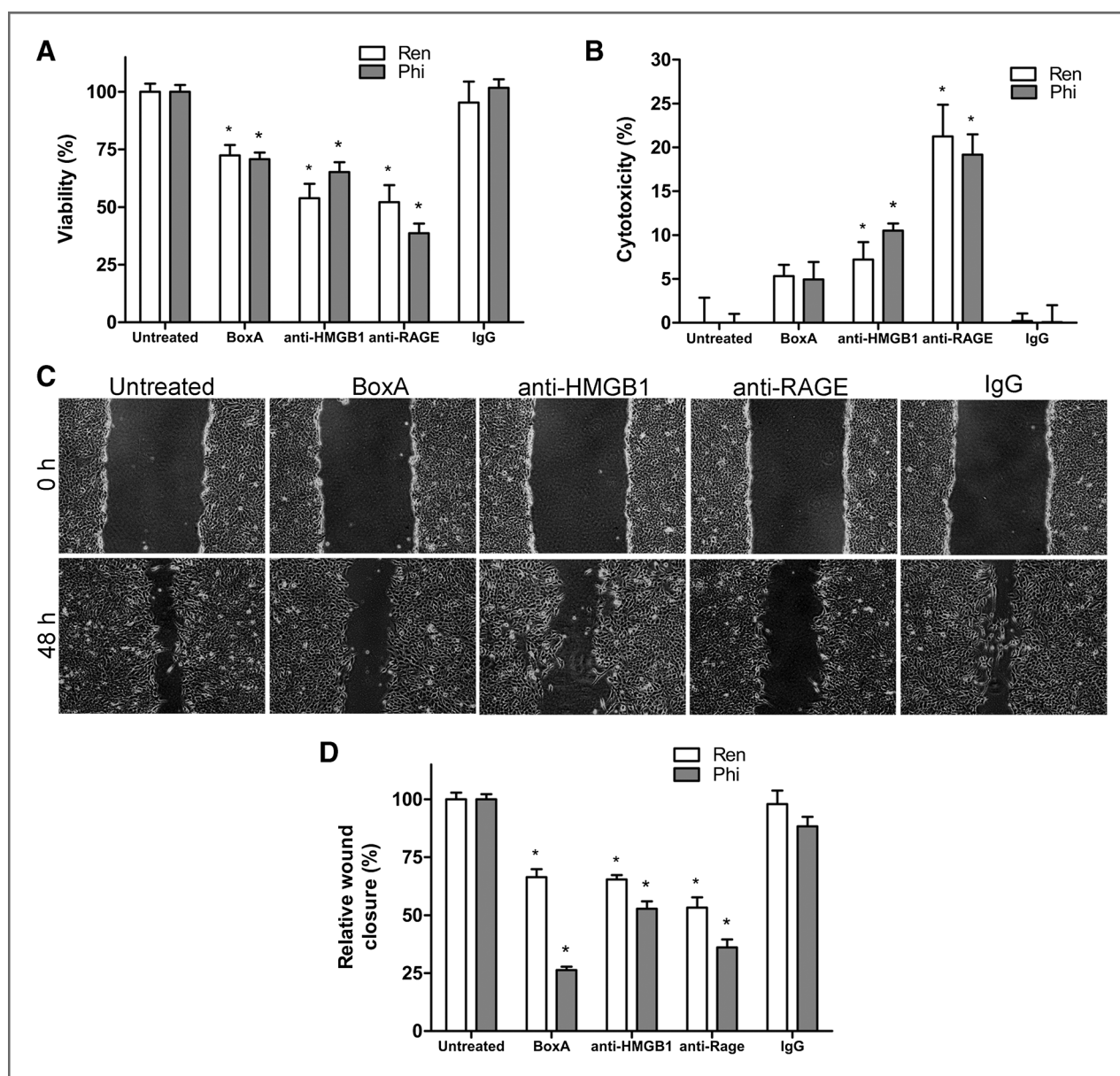


Figure 4. Malignant mesothelioma cells require HMGB1 for survival and migration. **A**, viability, determined by MTS assay. Inhibition of HMGB1 by BoxA or anti-HMGB1 or anti-RAGE antibodies substantially decreases the viability of REN and Phi cells. *, $P < 0.05$; treated versus untreated. **B**, cytotoxicity, determined by LDH assay. Both anti-HMGB1 and anti-RAGE antibodies induce substantial cytotoxicity in REN and Phi cells. Experiments were done in quadruplicate and conducted twice. *, $P < 0.05$; treated versus untreated. **C** and **D**, wound healing assay. One hour before scratching the monolayer, the cells were treated with either BoxA (100 ng/mL), anti-HMGB1 (1.0 μ g/mL), anti-RAGE (1.7 μ g/mL), or IgG control (1.7 μ g/mL). **D**, for quantification of wound closure, the scratched area covered by the cells after 48 hours was measured using ImageJ software and normalized to control. All HMGB1 antagonists reduced wound healing by REN (**C** and **D**) and Phi cells (**D**). Original magnification, $\times 40$. Figure shows representative images of wound healing (REN) from 3 experiments done in duplicate. *, $P < 0.05$; treated versus untreated. In all panels, error bars represent SEM.

Genes such as TNF- α and interleukin (IL)-1 α were upregulated, and genes downstream of TNFR1 and TNFR2 signaling were activated (Supplementary Fig. S9). Activation of these genes has been linked to malignant mesothelioma growth and invasion (1, 27).

These results indicated that HMGB1 sustains the main properties of the malignant phenotype (invasiveness and anchorage-independent growth) of mesothelioma cells.

Inhibition of HMGB1 *in vivo* reduces the growth of malignant mesothelioma xenografts and extends the mice survival

Our *in vitro* results suggested that HMGB1 supports the maintenance of the cancer phenotype of malignant mesothelioma, at least, in cells expressing high levels of HMGB1 that seem addicted to it. We validated this hypothesis in malignant mesothelioma xenografts in SCID mice (20, 21).

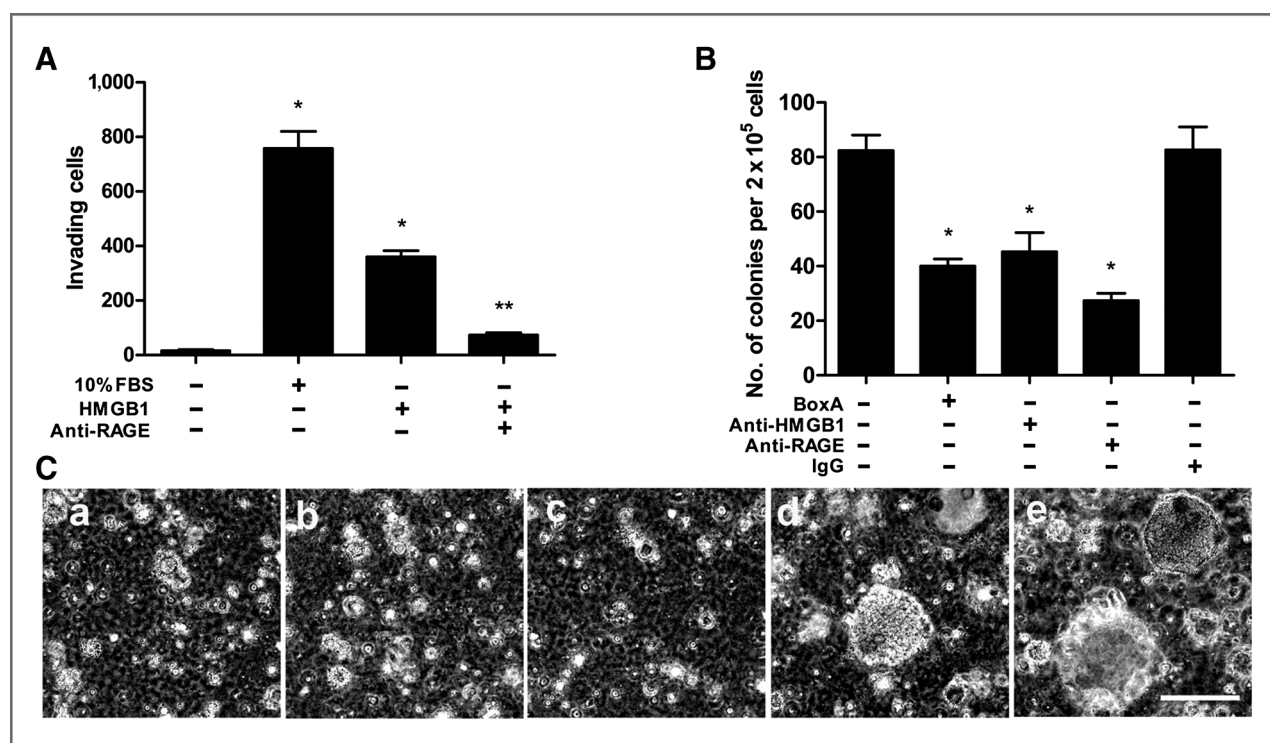


Figure 5. HMGB1 supports the malignant phenotype of mesothelioma cells. **A**, Matrigel invasion assay. Exogenous recombinant HMGB1 induces REN cells invasion. Anti-RAGE mAbs significantly inhibited HMGB1-induced cell invasion. Invading cells were counted using ImageJ software and represent mean values per field from at least 3 fields. *, $P < 0.05$; HMGB1 and 10% FBS versus 0% FBS. **, $P < 0.05$; HMGB1 alone versus HMGB1 plus anti-RAGE. **B** and **C**, anchorage-independent growth. REN cells treated with HMGB1 antagonists BoxA (**a**), anti-HMGB1 (**b**), and anti-RAGE (**c**) formed fewer (**B**) and smaller (**C**) colonies in soft agar than IgG (**d**) and untreated (**e**) controls. Original magnification, $\times 40$. Figure shows representative images from 2 experiments done in duplicate. Colonies larger than 0.1 mm in diameter were counted using ImageJ software. *, $P < 0.05$; treated versus IgG control. In all panels, error bars represent SEM. Scale bar, 0.5 mm.

Treatment with anti-HMGB1 mAb did not significantly inhibit tumor growth in mice inoculated with Mill cells, which are not "addicted" to HMGB1 (Supplementary Fig. S10A and B); however, anti-HMGB1 mAb significantly reduced tumor growth ($P < 0.05$; Fig. 6A and B) in mice inoculated with "HMGB1-addicted" REN cells and significantly extended animal survival by about 15% ($P < 0.05$; Fig. 6C).

Discussion

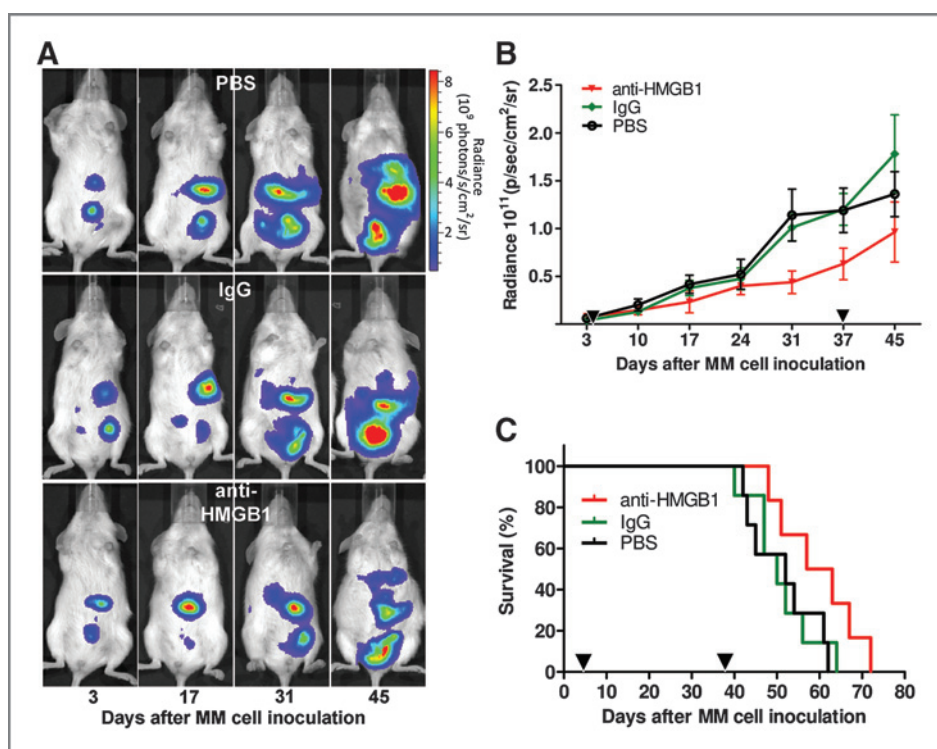
In previous studies we showed that, following asbestos and erionite exposure, HMGB1 is released by mesothelial cells undergoing programmed cell necrosis (9). HMGB1 starts a chronic inflammatory process that contributes to human mesothelial cells malignant transformation and mesothelioma development (5). Here we show that HMGB1 is secreted by malignant mesothelioma cells, and the levels of HMGB1 are elevated in the serum of malignant mesothelioma patients, making HMGB1 a potential malignant mesothelioma biomarker. HMGB1 supports malignant mesothelioma cell viability and hallmarks of malignant phenotype, such as tumor invasion and tumor cell proliferation, and treatment with HMGB1 inhibitors extended the

survival of mice xenografted with malignant mesothelioma cells.

Strong expression of HMGB1 was detected in the nuclei of the tumor cells of all malignant mesothelioma biopsies (31 of 31) tested. HMGB1 was also detected in the cytoplasm of the tumor cells in 26 of 31 malignant mesothelioma biopsies, a finding not observed in nearby normal stromal cells or in nonmalignant mesothelial cells.

HMGB1 is a biologically active protein, released by some immune cells (monocytes, macrophages, and dendritic cells) and other cells (pituitocytes, enterocytes, and hepatocytes) in response to specific stimuli, including LPS, TNF- α , IL-1, and IFN- γ (28–33). The presence of cytoplasmic HMGB1 staining suggested that malignant mesothelioma cells might also secrete HMGB1. Indeed, our results show that mesothelioma cells secrete HMGB1 into the extracellular space, as clearly shown by the detection of high concentrations of HMGB1 in the cytoplasm and in the tissue culture media of 6 of 7 malignant mesothelioma cell lines. The same 6 cell lines expressed high levels of RAGE, one of the main HMGB1 receptors. Moreover, we detected high levels of HMGB1 in the sera of all 20 malignant mesothelioma patients tested. The latter finding suggests that HMGB1 may be a biomarker of malignant mesothelioma, a hypothesis that we plan to test

Figure 6. Anti-HMGB1 mAb reduces tumor growth and extends survival in a mouse model of malignant mesothelioma. **A**, bioluminescence imaging of mice xenografted intraperitoneally with 5×10^5 REN/luc shows tumor progression. One representative mouse from each group is shown. **B**, tumor growth rate is reduced in mice injected with anti-HMGB1 mAb in comparison with controls. Mean \pm SEM of each group is shown ($n = 7$ per group). $P < 0.05$; 2-way ANOVA analysis. **C**, survival curve. Anti-HMGB1 mAb enhances animal survival in comparison with PBS and IgG control groups. Differences across groups ($P < 0.05$) were assessed by fitting a parametric model to the survival time data. Black triangles in **B** and **C** graphs represent first (day 4) and last (day 38) injections. MM, malignant mesothelioma.



in a clinical trial in an area of Cappadocia (Turkey), where a very high incidence of malignant mesothelioma is observed (3). The finding that HMGB1 secretion by malignant mesothelioma cells parallels the expression of the RAGE receptor suggests the occurrence of an autocrine mechanism of growth.

Other tumor cells, such as erythroleukemia, neuroblastoma, and colon cancer cells, have also been shown to secrete HMGB1 (34, 35). Once extracellular, HMGB1 triggers inflammation (26) and when secreted by tumor cells promotes their proliferation, migration, invasion, and neoangiogenesis (36–41). We show here that HMGB1 supports the proliferation, viability, motility, and invasiveness of malignant mesothelioma cells.

To test whether the withdrawal of HMGB1 would affect mesothelioma cells, we used different HMGB1 inhibitors: (i) BoxA, (ii) anti-HMGB1 mAb, and (iii) anti-RAGE mAb. Inhibition of the binding of HMGB1 to RAGE significantly diminished the viability of malignant mesothelioma cells expressing high levels of HMGB1.

We found that HMGB1 inhibitors impaired the anchorage-independent growth of mesothelioma cells, a hallmark of malignant transformation (42). Next, we tested whether HMGB1 inhibition could reduce the growth of tumors in malignant mesothelioma xenografted mice. Indeed, treatment with the anti-HMGB1 mAb in mice injected with human malignant mesothelioma cells that secrete high levels of HMGB1, such as REN, led to a significant decrease in mesothelioma growth and resulted in a significant extension in the survival of the xenografted mice. Notably, the only malignant mesothelioma cell line that secretes

low levels of HMGB1 was much less sensitive to HMGB1 inhibitors for its viability, motility, and invasiveness, consistent with our hypothesis that only tumor cells secreting HMGB1 constitutively become "addicted" to it. We cannot exclude the possibility that all malignant mesothelioma cells are primarily dependent on HMGB1, but some clones lose their dependence and their secretion ability as a secondary event.

In summary, we report that malignant mesothelioma cells become addicted to HMGB1 when it is upregulated together with its main receptor RAGE, and we propose that blockade of the HMGB1–RAGE interaction may represent a novel approach for malignant mesothelioma therapy.

Disclosure of Potential Conflicts of Interest

The University of Hawaii has filed for patents on HMGB1 and mesothelioma, on which H. Yang, M. Carbone, M.E. Bianchi, and H.I. Pass are named as inventors. M.E. Bianchi is founder and part owner of HMGBiotech.

Authors' Contributions

Conception and design: S. Jube, M.E. Bianchi, G. Gaudino, M. Carbone, H. Yang
Development of methodology: S. Jube, H. Yang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Jube, Z.S. Rivera, M.E. Bianchi, A. Powers, H.I. Pass
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Jube, M.E. Bianchi, A. Powers, E. Wang, I.S. Pagano, H.I. Pass, G. Gaudino, M. Carbone, H. Yang
Writing, review, and/or revision of the manuscript: S. Jube, M.E. Bianchi, A. Powers, E. Wang, H.I. Pass, G. Gaudino, M. Carbone, H. Yang
Study supervision: H. Yang

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