HMGB1 Promotes Drug Resistance in Osteosarcoma

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Abstract
Osteosarcoma is the most commonly occurring bone cancer in children and adolescents. Unfortunately, treatment failures are common due to the development of chemo-resistance, for which the underlying molecular mechanisms remain unclear. In this study, we implicate the DNA-binding protein HMGB1, which also exerts immunoregulatory effects in its secreted form, in the development of drug resistance in osteosarcoma. Anticancer agents including doxorubicin, cisplatin, and methotrexate each induced HMGB1 upregulation in human osteosarcoma cells, and RNA interference-mediated knockdown of HMGB1 restored the chemosensitivity of osteosarcoma cells in vivo and in vitro. Mechanistic investigation revealed that HMGB1 increased drug resistance by inducing autophagy, an intracellular self-defense mechanism known to confer drug resistance. We found that HMGB1 bound to the autophagy regulator Beclin1 and regulated the formation of the Beclin1–PI3KC3 [PI3KC3, phosphatidylinositol 3-kinase class 3] complex that facilitates autophagic progression. In addition, we found that interaction between HMGB1 and Beclin1 relied upon the autophagic complex ULK1–mAtg13–FIP200. Therefore, through its role as a regulator of autophagy, HMGB1 is a critical factor in the development of chemo-resistance, and it offers a novel target for improving osteosarcoma therapy. Cancer Res; 72(1); 230–8. ©2011 AACR.

Introduction
Osteosarcoma is the most common form of childhood and adolescent cancer, comprising 2-4% of all malignancies in pediatric patients and approximately 20% of all primary bone cancers (1). In the last 3 decades, the use of neoadjuvant chemotherapy in combination with surgery has increased the long-term survival rate of osteosarcoma patients. Cisplatin, doxorubicin, and methotrexate are commonly used anticancer agents, including radiotherapy and are significant obstacles to achieving favorable outcomes. Thus, the analysis of the molecular mechanisms underlying the resistance of osteosarcoma cancer cells to chemotherapy is essential for the development of novel treatment strategies for this disease.

High mobility group box 1 (HMGB1), a chromatin-binding nuclear protein, is expressed in many types of cells and is involved in several inflammatory diseases (4, 5). HMGB1 expression has also been linked to cancer development by interfering with several signaling pathways (6). Autophagy is a fundamental lysosomal process to confer stress tolerance and involves a series of steps including the formation of the phagophore, the autophagosome, and finally the autolysosome (7, 8). A number of studies have shown a critical role for autophagy in cancer development and therapy (9–11). Autophagy can promote or inhibit cancer cell growth depending on tumor types. For example, autophagy promotes pancreatic cancer growth (12) and inhibits breast carcinoma (13). In addition, there is a complicated relationship between autophagy and apoptosis following antitumor therapy that culminates in the determination of cancer cell fate. Recent studies suggest that HMGB1 plays a role in facilitating autophagy following cytotoxic insults including starvation (14–17). However, the mechanism and significance of HMGB1-mediated autophagy in tumor therapy still remains largely unknown.

In this study, we show that HMGB1-mediated autophagy contributes to chemotherapy resistance in osteosarcoma in vivo and in vitro. Our experimental data suggest an important role for HMGB1 in the regulation of autophagy through controlling the formation of Beclin 1–PI3KC3 [PI3KC3, phosphatidylinositol 3-kinase class 3] complex. These findings reveal novel potential targets for the treatment of osteosarcoma.

Materials and Methods
Cell culture, antibodies, and reagents
Human osteosarcoma cell lines (MG-63, SaOS-2, and U-2 OS) were obtained from the American Type Culture Collection...
(ATCC) and characterized according to ATCC instructions. These cell lines maintained in Eagle’s Minimum Essential Medium or McCoy’s 5a Medium Modified supplemented with 10% FBS. All cell lines were used within 20 passages. The antibodies to actin, Beclin 1, PIK3C3, C-PARP, ULK1, and P-ULK1 were obtained from Cell Signaling Technology. The antibodies to HMGB1 and mAtg13 antibody were obtained from Abcam. The antibodies to LC3, FIP200, and Atg7 were obtained from Novus. Other reagents were from Sigma.

**Cell viability analysis**
Cell viability was evaluated by the Cell Counting Kit-8 (Dojindo Laboratories) test as previously described (18). CCK-8 allows convenient cellular viability measurement using the enzymatic substrate, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt). WST-8 is reduced by dehydrogenase activity in cells to yield a formazan dye, which is soluble in the tissue culture media and detected colorimetrically at an absorbance of 450 nm. The amount of the formazan dye is directly proportional to the number of living cells.

**Western blot analysis**
Cell lysates were prepared with cell lysis buffer [20 mmol/L Tris-HCl, pH 7.5; 150 mmol/L NaCl; 1 mmol/L Na2EDTA; 1 mmol/L EGTA; 1% Triton; 2.5 mmol/L sodium pyrophosphate; 1 mmol/L β-glycerophosphate; 1 mmol/L Na3VO4; 1 μg/ml leupeptin; 1 mmol/L phenylmethylsulfonylfluoride (PMSF); and 1 mmol/L PMSF]. The lysates were cleared by centrifugation and total protein concentration was measured with the bicinchoninic acid assay Kit (Bio-Rad Laboratories). Proteins were resolved on a denaturing 10% SDS-PAGE gel and subsequently transferred to polyvinylidene fluoride membranes via semidyed transfer. The membrane was then blocked in 5% dried milk or 3% bovine globulin or specific antibodies in the presence of protein A sepharose beads. The beads were washed 3 times with RIPA buffer and the immune complexes were eluted from the beads and subjected to SDS-PAGE and immunoblot analysis as previously described (19, 20).

**Gene transfection and RNAi**
Transfection with pUNO1-HMGB1 cDNA (Invivogen) and/or HMGB1-shRNA, Beclin 1-shRNA, PIK3C3-shRNA, and Atg7 shRNA (Sigma) was carried out by the Lipofectamine 2000 Transfection Reagent (Invitrogen) according the manufacturer’s instructions. As a control experiment (Fig. 2F), another HMGB1-shRNA was obtained from Santa Cruz Biotechnology.

**Apoptosis assays**
The degree of apoptosis in cells was assessed by an Annexin V–propidium iodide Apoptosis Detection Kit (BD Pharminogen) by flow cytometric analysis as previously described (17). The degree of apoptosis in tissue was assessed with TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) kit from Roche Applied Science as previously described (18). Caspase 3 activity was analyzed by Colorimetric Caspase 3 Assay Kit (Merck) according the manufacturer’s instructions.

**Autophagy assays**
Formation of autophagic vesicles was monitored by endogenous LC3 aggregation in cell lines by LC3 antibody or mRFP–GFP–LC3 (Invitrogen). Autophagosomes have both mRFP and GFP signals, whereas the autolysosomes emit only mRFP signal because of the quenching of GFP in the acidic lysosomal environment (21, 22). The protein levels of LC3 and p62 was determined by Western blotting. Transmission electron microscopic (TEM) assessment of autophagosomes-like structures was carried out as previously described (23, 24).

**Mice xenograft models**
To generate murine subcutaneous tumors, 0.5 × 10⁶ MG-63 cells transfected with control or HMGB1-specific shRNA were injected subcutaneously to the right of the dorsal midline in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (The Jackson Laboratory) as previously described (25). One week after injection, the subcutaneous tumor size had reached a diameter of approximately 3 mm, and the mice then received i.p. injections of doxorubicin (5 mg/kg) twice a week thereafter. Tumor volumes were calculated by the following formula: length × width²/6 (18). All animal experiments strictly followed the guidelines of the Institutional Review Board.

**Statistical analysis**
In all experiments, unless otherwise indicated, data are reported as mean ± SEM in at least 3 replicates per group. Data were analyzed by 2-tailed Student t test or ANOVA least significant difference test, and P < 0.05 was considered significant.
Results

Anticancer agents promote HMGB1 expression in osteosarcoma cells

First, we assayed the effects of the anticancer agents doxorubicin (Dox), cisplatin (Cis), and methotrexate (Mtx) on the expression of HMGB1. These drugs promoted significantly enhanced expression of HMGB1 in the human osteosarcoma cell lines, MG-63, SaOS-2, and U-2 OS (Fig. 1A). Moreover, this effect was time dependent in the case of cisplatin in cell lines (Fig. 1B). In addition, a protein biosynthesis inhibitor cycloheximide (Chx) inhibited chemotherapy-induced HMGB1 protein expression (Fig. 1C). Real-time PCR revealed that HMGB1 mRNA was increased after treatment with these anticancer agents (Fig. 1D). These findings show that HMGB1 is upregulated during chemotherapy in osteosarcoma cells.

Suppression of HMGB1 increases sensitivity to chemotherapy in vitro

To explore the potential role for HMGB1 in the regulation of cell death in osteosarcoma cells, a target-specific shRNA from Sigma against HMGB1 was transfected into MG-63 and SaOS-2 cells. HMGB1 shRNA transfection led to a significant decrease of both HMGB1 protein and mRNA in these cells (Fig. 2A). Knockdown of HMGB1 expression in these cells rendered them significantly more sensitive to doxorubicin- and cisplatin- and methotrexate-induced cell injury (Fig. 2B) and this was associated with high levels of apoptotic cell death (Fig. 2C) and an increase in cleaved PARP (Fig. 2D). Cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis. Moreover, these anticancer agents induced the activation of the proapoptotic protein caspase 3 to a greater extent after HMGB1 knockdown (Fig. 2E). Moreover, addition of the pan-caspase inhibitor ZVAD-FMK reversed the increased caspase 3 activity (Fig. 2F). In addition, knockdown of HMGB1 in MG-63 cells by another HMGB1 shRNA from Santa Cruz Biotechnology also increased sensitivity to anticancer agent–induced cell injury and apoptosis (Fig. 2F). These data suggest that HMGB1 increases the resistance of osteosarcoma cells to a variety of cytotoxic agents.

Overexpression of HMGB1 increases resistance to chemotherapy in vitro

To further characterize the role of HMGB1 in osteosarcoma cells after chemotherapy, we transfected MG-63 and SaOS-2 osteosarcoma cells with full-length human HMGB1 cDNA (Fig. 3A). These HMGB1-overexpressing cells became resistant to apoptosis induced by doxorubicin, cisplatin, and methotrexate (Fig. 3B), confirming a potential antiapoptotic role for HMGB1 in osteosarcoma cells. Autophagy and apoptosis are at times triggered by common upstream signals and can result in both initiation of both processes simultaneously (26). Microtubule-associated protein light chain 3 (LC3) is widely used to measure autophagy (21). Overexpression of HMGB1 increased LC3 puncta formation after chemotherapy treatment (Fig. 3B). To explore whether autophagy mediates the effects of HMGB1–mediated resistance to the apoptotic response after treatment with anticancer agents, we knocked down PI3KC3, Beclin 1, and Atg7 (Fig. 3C), which are critical autophagic regulators in mammalian cells. Silencing of these genes in MG-63 cells inhibited HMGB1 overexpression–induced LC3-II formation and prevented autophagic p62 degradation (Fig. 3C). Baflomycin A1, an inhibitor of late phase autophagy (21), increased overexpression of HMGB1–induced LC3-II formation, whereas 3-methyladenine, an inhibitor of early-phase autophagy (21), inhibited overexpression of HMGB1–induced LC3-II expression.
This finding suggests that overexpression of HMGB1 increases LC3 turnover but does not impair degradation of LC3. Moreover, silencing of these genes reversed HMGB1-induced protection against chemotherapy (Fig. 3E) accompanied by decreased autophagy (Fig. 3F) and increased apoptosis (Fig. 3G). These findings suggest that autophagy is required for this HMGB1-mediated antiapoptotic effect.

**HMGB1 regulates autophagy during chemotherapy in osteosarcoma cells**

To explore whether HMGB1 regulates autophagy in osteosarcoma cells, we detected LC3-I to LC3-II conversion by immunoblot analysis or LC3 puncta formation by fluorescent imaging analysis. Knockdown of HMGB1 inhibited chemotherapy-induced expression of LC3-II (Fig. 4A). When autophagosomes form, LC3 is lipidated and recruited to the autophagosomal membrane. Accumulation of LC3-II is observed in the presence of bafilomycin A1 (Fig. 4A). Knockdown of HMGB1 inhibited accumulation of LC3 puncta in osteosarcoma cells by LC3 antibody or mRFP-GFP-LC3 construct (Fig. 4B). Moreover, ultrastructural analysis revealed that HMGB1 shRNA cells exhibited few autophagosomes during chemotherapy compared with control shRNA cells (Fig. 4C). To further investigate whether HMGB1 influences autophagic flux, we evaluated the expression of SQSTM1/sequestosome 1 (p62), which forms protein aggregates that are degraded by autophagy (27, 28). Indeed, knockdown of HMGB1 inhibited autophagic p62 degradation (Fig. 4A). These findings support a critical role for HMGB1 in the regulation of autophagy in osteosarcoma cells. Rapamycin induces autophagy by inhibiting the protein mTOR. Consistently with previous studies (29, 30), rapamycin pretreatment protected against doxorubicin-induced apoptosis in HMGB1 wild-type cells (Fig. 4D). However, rapamycin conferred less protection in HMGB1 knockdown cells due to diminished autophagic capacity (Fig. 4E). These findings suggest that HMGB1 is an important regulator of autophagy-mediated cell survival.
HMGB1 regulates formation of Beclin 1–PI3KC3 complex but not ULK1–mAtg13–FIP200 complex in autophagy

The process of mammalian autophagy is divided into several principal steps: initiation, nucleation, elongation, closure, maturation, and finally degradation or extrusion (7). These steps are themselves mediated by a core family of proteins—the ATG proteins (31). To explore the potential mechanism of HMGB1-mediated autophagy, we first analyzed the early autophagic signaling event of ULK1 complex formation. ULK1 is essential for autophagy induction and is comprised of a large complex that includes a mammalian homologue of Atg13 (mAtg13) and the scaffold protein, FIP200 (an orthologue of yeast Atg17). Knockdown of HMGB1 does not affect formation of ULK1–mAtg13–FIP200 complex and phosphorylation of ULK1 at Ser55 following doxorubicin treatment (Fig. 5A). However, HMGB1 knockdown does influence the formation of the Beclin 1–PI3KC3 complex (Fig. 5B), which mediates vesicle nucleation in autophagy. Consistent with previous studies (14, 32), endogenous HMGB1 formed a complex with Beclin1 which was detected by coimmunoprecipitation in osteosarcoma cells (Fig. 5C). Moreover, knockdown of ULK1 or FIP200 inhibited the interaction between HMGB1 and Beclin 1 (Fig. 5C) and increased sensitivity to anticancer agent–induced cell apoptosis (Fig. 5D). These studies suggest that HMGB1 is a downstream signal from ULK1–mAtg13–FIP200 complex formation and facilitates autophagy in osteosarcoma cells by interacting with Beclin 1. Inhibition of this pathway in autophagy will increase drug sensitivity.

Suppression of HMGB1 increases sensitivity to chemotherapy in vivo

To test whether targeted knockdown of HMGB1 also increased sensitivity to chemotherapy in vivo, we inoculated NOG/SCID mice with MG-63 tumor cells that had previously been transfected with HMGB1-specific shRNA. Beginning at day 7, mice were treated with doxorubicin. The growth of HMGB1 knockdown tumor cells was significantly inhibited compared with control shRNA–transfected tumors after treatment (Fig. 6A). We observed that tumor cells transfected with HMGB1-specific shRNA showed decreased autophagy and increased apoptosis in vivo in response to therapy compared with control shRNA-transfected tumor cells (Fig. 6B and C). Together, these results show that HMGB1 is important in modulating drug resistance in osteosarcoma cells in vivo.
Discussion

Many osteosarcoma patients develop resistance to chemotherapy drugs, and this phenomenon has been attributed to different mechanisms including dysfunctional membrane transport, resistance to apoptosis, and the persistence of stem cell–like tumor cells. In this study, we showed that HMGB1-mediated autophagy is a significant contributor to drug resistance in osteosarcoma. Inhibition of HMGB1 or autophagy increased the drug sensitivity of osteosarcoma cells.

HMGB1 is a nonhistone chromatin protein that acts as a damage associated molecular pattern molecule (DAMP) when released into the extracellular milieu (33). As a redox sensitive protein, HMGB1 has been implicated as an important factor in both sterile inflammation and infection as well as in cancer, diabetes, and neurodegenerative diseases (5, 34). HMGB1 is a bone active cytokine and regulates the bone microenvironment (35, 36). For example, HMGB1 acts as a chemotactic agent to osteoclasts and osteoblasts during endochondral ossification (36). Overexpression of HMGB1 is associated with each of the hallmarks of cancer including unlimited mitosis, the ability to recruit new vascular structures, evasion of programmed cell death (apoptosis), avoiding immune destruction, self-sufficiency in growth signals, insensitivity to inhibitors of growth, tumor-promoting inflammation, dysregulation of cellular energetics, genome instability and mutation, and tissue invasion and metastasis (37–39).

One of the mechanisms by which chemotherapeutics destroy cancer cells is by inducing cell death. We found that doxorubicin, cisplatin, and methotrexate promote enhanced expression of HMGB1 in human osteosarcoma cells. Moreover, knockdown of HMGB1 by shRNA increased cell death and...
suppressed osteosarcoma growth in vivo and in vitro. In addition, HMGB1 is also a negative regulator of drug-induced cell death in other tumors such as leukemia (16), colon cancer (17) and prostate cancer (40). As a DAMP, release of HMGB1 by dead and dying cells triggers inflammation (41) and the immune response (4). Thus, release of HMGB1 by dying tumor cells is helpful to host dendritic cells which process and present tumor antigens to immune effector cells (42). In addition to the extracellular release HMGB1, its redox status is critical to regulating cell death and survival in cancer cells. Reduced exogenous HMGB1 protein promotes cell survival, whereas oxidized HMGB1 induces apoptosis (17).

Cancer cells respond to chemotherapy in a variety of ways ranging from the activation of survival pathways to the initiation of cell death. Increased autophagy is observed in osteosarcoma cells when exposed to chemotherapy drugs (43, 44).

**Figure 5.** ULK1–mAtg13–FIP200 complex formation regulates HMGB1–Beclin 1 complex formation in osteosarcoma cells. A–C, MG-63 cells were transfected with HMGB1 shRNA (A and B) or ULK1 shRNA (C) or FIP200 shRNA (C) for 48 hours and then were treated with Dox (0.2 μg/mL) for 24 hours. Cells were then assayed for protein expression levels as indicated by immunoprecipitation (IP) or Western blotting (IB) as described in Materials and Methods. All data are representative of 2 or 3 experiments. D, indicated MG-63 cells were treated with Dox (0.2 μg/mL), Cis (20 μmol/L), and Mtx (50 μmol/L) for 24 hours and then apoptosis was analyzed by measuring Annexin V–positive cells by flow cytometry (n = 3; *, P < 0.05).

**Figure 6.** Suppression of HMGB1 increases sensitivity to chemotherapy in vivo. A, NOD/SCID mice were inoculated with 0.5 × 10^6 MG-63 tumor cells following transfection of control or HMGB1-specific shRNA and treated with Dox (5 mg/kg) beginning at day 7. Tumors volumes were calculated for 28 days (n = 6; *, P < 0.05). B, on day 28 in the experiments described in (A), apoptosis and autophagy in tumor samples were assayed by TUNEL (red) or LC3 (green) stain respectively.
In general, autophagy is a “programmed cell survival” mechanism because cells using autophagy to prevent the accumulation of damaged or unnecessary components, but also functions to facilitate the recycling of these components to sustain homeostasis. We found that inhibition of autophagy increases osteosarcoma cell death and reverses HMGB1-mediated drug resistance. Recently, a systematic chemical study on cells exposed to 1,400 compounds showed that no single cytotoxic agent can induce cell death by autophagy (45), confirming that autophagy is mostly a cytoprotective mechanism (8). Thus, autophagic cell death may be a misnomer (46).

It has been reported that HMGB1 is a critical regulator of autophagy. Cytosolic HMGB1 is an inducer of starvation-mediated autophagy by binding to Beclin 1 in mouse embryonic fibroblasts and colon cancer cells (14). In addition, reduced extracellular HMGB1 induces autophagy through the receptor for advanced glycation endproducts (RAGE) in colon and pancreatic cancer cells (17). Furthermore, nuclear HMGB1 regulates HSPB1 gene expression (15). Phosphorylation of HSPB1 is necessary for the regulation of the actin cytoskeleton, which mediates the vesicular transport required for autophagy in response to mitochondrial injury. Here, we found that knockdown of HMGB1 decreased LC3 II levels, LC3 puncta formation, and p62 degradation which was associated with a decreased number of membraned autophagosomes by detected by TEM in osteosarcoma cells. Notably, we showed that the ULK1–FIP200 complex is required for the interaction between HMGB1 and Beclin 1, which then promotes Beclin 1–PINK3 complex formation. However, the assembly of the Beclin 1 complexes is complex and seems to differ in a cell- or tissue-dependent fashion (47). AMP-activated protein kinase (AMPK) is a key molecular player in energy homeostasis and is important for the activation of ULK1 (48). It is unknown whether AMPK and other kinases are involved in the regulation of the interaction between HMGB1 and Beclin 1.

In conclusion, we showed that chemotherapy-induced HMGB1 expression in osteosarcoma cells promoted autophagy, which inhibited apoptosis and increased drug resistance. Our in vitro and in vivo xenograft studies showed that suppression of HMGB1 expression significantly increased drug sensitivity of osteosarcoma cells. Thus, we believe these data support an investigation of HMGB1 as a strategic target for osteosarcoma therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This work was supported by grants from The National Natural Sciences Foundation of China (30973234 to L. Cao) and a grant from the University of Pittsburgh (D. Tang)

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Received June 16, 2011; revised October 24, 2011; accepted November 11, 2011; published OnlineFirst November 18, 2011.

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www.aacrjournals.org Cancer Res; 72(1) January 1, 2012 237

Published OnlineFirst November 18, 2011; DOI: 10.1158/0008-5472.CAN-11-2001
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