HMGB1 regulates autophagy through increasing transcripational activities of JNK and ERK in human myeloid leukemia cells

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INTRODUCTION

High-mobility group box 1 protein (HMGB1), one of the best characterized damage-associated molecular pattern (DAMP) molecule, is a chromatin associated nuclear protein. Intracellularly, HMGB1 functions as an architectural chromatin-binding factor and promotes assembly of the proteins including p53, p73, Rel/NF-κB and estrogen receptor on specific DNA targets. Extracellularly, HMGB1 through interacting with the receptors RAGE and TLRs contributes to several biological functions including inflammation, cell migration, cell differentiation and tumor metastasis (1). Current studies reveal that HMGB1 plays an important and central role in cancer (2). The upregulation of HMGB1 mRNA expression was found in tumors including gastrointestinal stromal tumor, skin tumor, colon cancer, breast cancer, lung cancer, prostate cancer, cervical cancer, gastric cancer, hepatocellular carcinoma, leukemia, lymphoma and melanoma. Therefore, targeting the HMGB1 ligand or its receptor represents an important potential application in cancer therapeutics.

Autophagy occurs at low basal levels in all cells and is postulated to play a housekeeping role (3). It is part of the normal catabolic process needed for homeostasis, which is in the quality control of cellular components by recycling long-lived proteins and dysfunctional organelles, and also keeps cell prolonged survival by providing substrates under metabolic stresses. Autophagy occurs in response to disordered micro-environment stimuli for H2O2, rapamycin, ER stress, mitochondrial toxins, hypoxia, abnormal cell growth, and nutrient deprivation (4). It is associated with multiple disease states, involving neurodegenerative disease, infection, heart disease, autophagic cell death, and cancer (5). Although the paradoxical dual effect about autophagy on tumor formation and growth awaits more verification, overwhelming investigations have suggested autophagy is an important resistance mechanism to chemotherapy in hematological malignancies (6).

Our resent studies have demonstrated that the serum levels of HMGB1 are significantly high in childhood lymphocytic leukemia (7, 8). Later data confirms HMGB1 is a direct activator of autophagy in leukemia cells through activating PI3KC3-MEK-ERK pathway (9), and autophagy is a potential mechanism for HMGB1-mediated chemotherapy resistance. However, the correlation between autophagy and HMGB1 in human myeloid leukemia cells remains unclear.

In this paper, our results suggested that HMGB1 over-expression rendered myeloid leukemia cells (K562 cells) resistant to conventional anticancer treatments through increasing autophagy rather than decreasing apoptosis. On the other hand, suppression of HMGB1 expression increased the sensitivity of leukemia cells to chemotherapeutic drugs. HMGB1 over-ex-
expression resulted in an increase in the formation of autophagosome and autophagolysosome fusion, in mRNA levels of Beclin-1, VSP34 and UVRAG which are key genes involved in mammalian autophagy, and in protein levels of p-Bcl-2 and LC3-II. The luciferase activity assays revealed that over-expression of HMGB1 increased the synergistic transcriptional activities of JNK and ERK, respectively, and the inhibition of HMGB1 down-regulated the transcriptional activities of JNK and ERK. The role of HMGB1 in transcriptional regulation of JNK and ERK required for autophagy provides a potential drug target for therapeutic interventions in myeloid leukemia.

RESULTS

The HMGB1 expression is associated with the progression of childhood chronic myeloid leukemia

We firstly determined mRNA levels of HMGB1 in four leukemia cell lines (HL60, K562, Jurkat, and Raji) by RT-PCR analysis. Levels of HMGB1 expression were high in all four human leukemia cell lines. In contrast, the expression levels of HMGB1 were noticeably low in non-blood cancer cell-lines (A549, Hela, and HepG2) and the bone marrow mononuclear cells (BMMCs) from normal children. These results suggested that the expression of HMGB1 in leukemia tumorigenesis was up-regulated (Fig. 1A).

To evaluate the clinical relevance, we determined the HMGB1 protein expression levels in serum from normal healthy subjects, children with newly diagnosed chronic myeloid leukemia (CML) and remission after treatment. The expression of HMGB1 was significantly lower in Control group (59.06 ± 15.76 ug/L) and remission group (62.04 ± 28.98 ug/L) than that in the refractory group (345.00 ± 123.42 ug/L), P < 0.05. The difference of HMGB1 expressions between the control and remission groups was not significant (P > 0.05) (Fig. 1B). These results suggested that HMGB1 might be involved in the

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Fig. 1. (A) HMGB1 was over-expressed in blood cancer cell lines. RT-PCR analyses of HMGB1 and β-actin in various human cancer cell lines and the bone marrow mononuclear cells (BMMCs) from normal children, as indicated. (B) Expression of HMGB1 in serum of different groups. The expression of HMGB1 is significantly lower in Control group (59.06 ± 15.76 ug/L) and remission group (62.04 ± 28.98 ug/L) than that in the refractory group (345.00 ± 123.42 ug/L), P < 0.05. The difference of HMGB1 expressions between the control and remission groups is not significant (P > 0.05).

Fig. 2. HMGB1 over-expressed by plasmid transfection and knocked down by siRNA in K562 cells as indicated and then treated with HBSS (Hank’s), 3 hours later, capase-9 inhibitor for apoptosis was used (B and C) or not (A). (A) HMGB1 over-expression rendered K562 cells resistant to anti-cancer drug-induced apoptosis. K562 cells treated as indicated and then treat with VCR (1 ug/ml), ADM (1 ug/f), VP-16 (1 ug/ml), AS2O3 (5 um), PBS treat as control group. Cell viability was examined at 24 h (n = 3, *P < 0.05), (B) The analysis of changes in cell cycle was quantified by flow-cytometry after PI staining in K562 cells treated 48 h after 3-h pretreatment with HBSS, there are no differences between HMGB1+ vs HMGB1- group. Values were given as mean ± SD. A: HMGB1+; B: HMGB1-. (C) Autophagic vacuoles were labeled with 0.05 mmol/L monodansylcadaverine (MDC) in phosphate-buffered saline (PBS) at 37°C for 10 min. The fluorescent density and the MDC-labeled particles in K562 cells were significantly higher in HMGB1+ than in HMGB1- group. (x×400 magnifications). A: HMGB1+; B: HMGB1-. Data are the means SEM of three separate experiments.
development of childhood chronic myeloid leukemia.

**HMGB1 decreases the sensitivity of K562 cells to anti-cancer drug induced death through up-regulating the autophagy pathway**

We examined the cell viability by CCK8 to explore the potential role of HMGB1 in the regulation of cell sensitivity following treatment with chemotherapy. HMGB1 over-expression rendered leukemia cells resistant to anti-cancer drug including VCR, ADM, VP-16 and AS2O3 compared with the cells treated with a target-specific siRNA duplex against HMGB1 (Fig. 2A), indicating a potential prosurvival role of HMGB1 in cells exposed to chemotherapy.

There are two processes to occur simultaneously during cell death. The classical characteristics of Type II programmed cell death (apoptosis) distinct from Type I programmed cell death (apoptosis) is whether it requires caspase activation or DNA fragmentation or not. To explore the potential mechanism of HMGB1-mediated drug resistance in K652, flow-cytometry experiment was performed, and the results showed that there were no apoptosis differences between HMGB1+ vs HMGB1− group (Fig. 2B).

It has been reported that MDC is a specific marker for autophagic vacuoles. When the cells were viewed under a fluorescence microscope, MDC-labeled autophagic vacuoles appeared as distinct dots like structures distributing in cytoplasm or in per nuclear. Interestingly, the fluorescent density and MDC-labeled particles of K562 cells were higher in HMGB1+ treatment group than in HMGB1− (Fig. 2C), suggesting that HMGB1 induces formation of MDC-labeled vacuoles, and it was the autophagy but not apoptosis that played a role required for HMGB1-mediated drug resistance in leukemia cells (Fig. 2C).

**HMGB1 induces autophagy in leukemia cells**

Transmission electron microscope (TEM) remains an important tool for detecting autophagosomes and provides significant insights to the extent of on-going autophagy in cells (10). At the ultrastructural level, Type II autophagosomes are defined as double-membraned structures containing undigested cytoplasmic contents, which have not fused with a lysosome. In contrast, Type II autophagolysosomes are hybrid organelles generated by the fusion of autophagosomes and lysosomes, which have single limiting membranes and contain cytoplasmic materials at various stages of degradation. Ultrastructural analysis revealed that K562 cells exhibited more Type II autophagosomes and Type II autophagolysosomes after HMGB1+ treatment when compared with the HMGB1− (Fig. 3A). These results indicated that HMGB1 was indeed an important activator of autophagy.

Semi-Quantitative RT-PCR results revealed that mRNA levels of the key markers Beclin-1, VSP34 and UVRAG in mammalian autophagy were increased following the over-expression of HMGB1, while mRNA levels of Beclin-1, VSP34 and UVRAG were decreased after inhibition of HMGB1 by siRNA (Fig. 3B). Western blotting analyses confirmed that the conversion of LC3-I to LC3-II and p-Bcl-2 were increased following the over-expression of HMGB1 (Fig. 3C).

**HMGB1 increases JNK and ERK-mediated transcriptional activation**

We performed reporter gene assays to measure the modulation of
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Fig. 4. (A) HMGB1 increased JNK transcriptional activity, which was reversed or abated by siRNA knockdown of HMGB1 in control or HMGB1 overexpression cells. (B) HMGB1 increased ERK transcriptional activity, which was reversed or abated by siRNA knockdown of HMGB1 in control or HMGB1 overexpression cells. COS-7 cells were transfected with reporter plasmid and the corresponding plasmids were shown in the gures. Forty-eight hours later, the luciferase activity assay was performed. The data are means of three repeats in a single experiment after normalization for β-galactosidase activity. Each experiment was performed at least three times.

JNK and ERK by HMGB1 protein in the cells. COS-7 cells were co-transfected with the expression plasmids pCMV-Tag2C-HMGB1, pJNK-Luc and pERK-Luc. Over-expression of HMGB1 signitantly increased the endogenous JNK-luciferase activity (Fig. 4A).

We then tested the role of HMGB1 in the transcriptional activity of ERK. As observed in Fig. 4B, over-expression of HMGB1 strongly increased the endogenous transcriptional activity of ERK. Then HMGB1 was silenced by siRNA in COS-7 cells and the results showed that the JNK and MEK luciferase activities were decreased when co-transfected with RNAi plasmid, suggesting that siRNA targeting HMGB1 abolished the transcriptional suppression of HMGB1. Taken together, our results suggested that HMGB1 may act as a transcriptional activator in the MAPK signaling pathway to mediate cellular functions.

DISCUSSION

Over-expression of HMGB1, the best characterized DAMP, is associated with all of the central hallmarks of cancer (11). The mechanisms by which it contributes to carcinogenesis, and therapeutic strategies based on targeting HMGB1 become focus in biochemistry, molecular biology, and clinical field.

Recently, a rapid growing body of literature supports the function of HMGB1 as a direct activator of autophagy in leukemia cells and autophagy is a potential mechanism for HMGB1-mediated chemotherapy resistance. However, the mechanism underlying HMGB1 induces autophagy in human myeloid leukemia cells remains unclear. In this study, our experiments involving cellular models and clinical samples demonstrate that mRNA level of HMGB1 is high in leukemia cells and it is involved in the progression of childhood chronic myeloid leukemia. Because HMGB1 can decrease the sensitivity of human myeloid leukemia cells K562 to anti-cancer drug induced death, developing new therapeutic strategies for childhood CML based on the HMGB1 regulation of autophagy is worth to be explored.

A series of investigations have confirmed that Beclin1 (the first mammalian autophagy protein to be described) binds to the anti-apoptotic Bcl-2 protein through its BH3 domain. The kinases of JNK or ERK signaling pathway mediated Bcl-2 at three different sites in its BH3 and BH4 domains. In response to starvation-induced signaling, JNK/ERK are activated, which leads to the dissociation of Bcl-2 from Beclin-1. The separation of Beclin1-Bcl-2 is an important molecular event which resulted in UV irradiation resistance-associated gene (UVRAG) recruited to class III phosphatidylinositol-3 kinase (vacuolar sorting protein 34, VPS34), and the complex "Beclin-VPS34-UVRAG" is crucial for the induction of mammalian autophagy (12). HMGB1 has also been shown to interact with and enhance the activities of a number of transcription factors implicated in cancer development such as protein kinase B (AKT), mitogen activated protein kinases (MAPKs), and NF-B (1). Our experiments with mouse embryo fibroblast cells have shown that endogenous HMGB1 is a novel Beclin-1 binding protein active in autophagy. It can regulate activation of mitogen-actives protein kinase (MAPK), which results in ERK1/2-mediated phosphorylation of Bcl-2 and subsequently dissociation of the Beclin-1-Bcl-2 complex (13). Here, our experimental results suggest that HMGB1 regulates autophagy through increasing transcriptional activities of JNK and ERK in human myeloid leukemia cells, because: 1) cellular level: MDC is a specific marker for autophagic vacuoles, and TEM remains one of the most widely used and sensitive techniques to detect the presence of autophagic vesicles. HMGB1 induces formation of MDC-labeled vacuoles, and more Type I autophagosomes and Type II autophagolysosomes in K562 cells vividly confirmed the autophagy occurring from morphology level; 2) protein level: LC3 is considered a marker for autophagy when it is proteolytically processed and conjugated to phosphatidylethanolamine (LC3-II) (14). Immunoblot analysis has shown that exogenous HMGB1 increases the conversion of LC3-I to LC3-II and induces autophagy in K562 cells; 3) gene level: the expression of Beclin-1, VSP34 and UVRAG which are key genes involved in mammalian autophagy are increased following the over-expression of HMGB1; 4) mo-
K562 cells were prepared and treated as described, 48 hours after analysis of the cell cycle by flow-cytometry and used to determine the concentration of HMGB1 in the sample. A standard curve was plotted from measurements made with the standard solutions according to the manufacturer’s instructions. A standard curve from Shino-Test Corporation (Sagamihara-shi, Kanagawa, Japan) was based on the measurement of the DNA content of nuclei labeled with propidium iodide (PI), according to manufacturer’s instructions. Treated cells were trypsinized (250 μl of trypsin buffer) for 10 min at room temperature, and then trypsin inhibitor (200 μl) and RNase buffer were added and allowed to react for 10 min at room temperature. Finally, propidium iodide stain solution (200 μl) was added and incubated for 10 min in the dark on ice. Samples were immediately analyzed in the flow-cytometer (COULTER EPICS XL), and the obtained results analyzed by the Cell Quest software (Becton-Dickinson). The experiment was performed three times, and the ratio of cells in the G0/G1, intra-S, and G2/M phases were expressed as mean ± SD.

**MATERIALS AND METHODS**

**Clinical data**
(1) Untreated group: from May 2009 through June 2010, 12 children with newly diagnosed chronic myeloid leukemia (CML) in our hospital. This cohort of patients (median age at diagnosis 9 years, range 3 months-12 years), including males 8 and females 4, were diagnosed based on FAB (French-American-British classification) standards, and according to the declaration of MIC (Morphology, Immunology and Cytogenesis). (2) Mitigation groups: with the same group of untreated. (3) Control group: 12 cases of healthy children were as control, the median follow-up time was 5 years (range 1-12 years). (4) Clinical specimen collection and processing: all patients were treated by the protocol which was recommended by Chinese Medical Association, all children provided a 2 ml peripheral blood sample collected into ethylenediaminetetra-acetic (EDTA) tubes, and isolated serum were frozened at −70°C for experiment.

**ELISA**
Human HMGB1 released into cell culture supernatants was evaluated using enzyme-linked immunosorbent assay (ELISA) kits from Shino-Test Corporation (Sagamihara-shi, Kanagawa, Japan) according to the manufacturer’s instructi-ons. A standard curve was plotted from measurements made with the standard solutions and used to determine the concentration of HMGB1 in the sample.

**Cell culture, transfection and RNAi**
K562 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco, Tulsa, USA) and 1% penicillin/streptomycin at 37°C in a 5% humidified CO2 atmosphere. Cells were transfected with pEGFP-N1-HMGB1 or pEGFP-N1 using so-fast TM (Xiamen Sunma Biotechnology Co, Ltd.) according to the manufacturer’s directions. Human HMGB1 siRNA or control-siRNA was transfected into cells using X-treme GENE siRNA reagent (Roche Applied Science, Sweden) according to the manufacturer’s instructions.

**Analysis of the cell cycle by flow-cytometry**
K562 cells were prepared and treated as described, 48 hours after transient transfection, the percentage of cells in each phase of the cell cycle was analyzed by flow-cytometry, using the Cycle TEST PLUS DNA Reagent Kit (BD Pharmingen, San Jose, CA, USA), which was based on the measurement of the DNA content of nuclei labeled with propidium iodide (PI), according to manufacturer’s instructions. Treated cells were trypsinized (250 μl of trypsin buffer) for 10 min at room temperature, and then trypsin inhibitor (200 μl) and RNase buffer were added and allowed to react for 10 min at room temperature. Finally, propidium iodide stain solution (200 μl) was added and incubated for 10 min in the dark on ice. Samples were immediately analyzed in the flow-cytometer (COULTER EPICS XL), and the obtained results analyzed by the Cell Quest software (Becton-Dickinson). The experiment was performed three times, and the ratio of cells in the G0/G1, intra-S, and G2/M phases were expressed as mean ± SD.

**MDC staining of autophagic vacuoles**
MDC staining of autophagic vacuoles was performed for autophagy analysis as previously described. K562 cells were divided into pEGFP-N1-HMGB1 or HMGB1 siRNA treated group. The cells were incubated for 48 h and starved in the Hank’s for 3 h; capase 9 was used for inhibiting apoptosis. Autophagic vacuoles were labeled with 0.05 mmol/L MDC in PBS at 37°C for 10 min. After incubation, the cells were washed three times with PBS and immediately analyzed under a fluorescence microscope (E-400 Nikon, Japan). Fluorescence of MDC was measured at the excitation wavelength 380 nm with an emission filter at 530 nm.

**Cell viability assay**
Cells were plated at a density of 5 × 10⁴ cells/well in 96-well plates in 100 μl medium. After treatment, cell viability was evaluated with Cell Counting Kit-8 (CCK8) (Dojindo, Japan) according to manufacturer’s instructions. In parallel analysis of cell viability by trypan blue exclusion assay was performed.

**TEM of transfected cell**
K562 cells were transfected with either pEGFP-N1- HMGB1 or HMGB1 siRNA as described above. Thirty-six hours post-transfection and cultured in the Hank’s for 3 hours, capase 9 was used for inhibiting apoptosis occur. Cells were harvested and washed twice with 1× PBS followed by an additional wash in 0.1 M sodium cacodylate. To prepare thin sections, cell pellets were first fixed with 2.5% glutaraldehyde for 24 h and then washed three times with 0.1 M sodium cacodylate. After washing, the samples were post-fixed with 1% OsO₄ for 2 h, followed by three rinses. The samples were then subjected to increasing concentrations of acetone for dehydration. Immediately following the application of 70% ethanol, en bloc staining was added to the samples for 30 min before embedding in Epon 812 resin. Ultrathin sections (50 nm) were acquired and stained with uranyl acetate and lead citrate, then examined by electron microscopy using an H-7500 1200EX transmission electron microscope. The quantification of autophagosomes and autophagolysosomes was performed as
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previously described.

RNA isolation and Semi-Quantitative RT-PCR analysis
Total RNA was extracted from K562 cells after treated, using TRIZOL reagent (Invitrogen) following the manufacturers instructions and was quantified by absorbance at 260 nm, its integrity was determined by examining the 28S and 18S rRNA bands in ethidium bromide stained agarose gels. Then subjected to reverse transcription amplification were performed using the Access RT-PCR System according to the manufacturer’s protocol. PCR conditions were as follows: initial denaturation at 95°C for 4 min, followed by 30 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 30 s, then by an 8 min elongation at 72°C. All primer sets (Supplemental Table 1) were designed to span at least one intron in order to distinguish reverse transcribed DNA from DNA contamination; PCR fragments were analyzed on a 1.5% agarose gel and visualized by ethidium bromide staining.

Western blot analyses
The treated cells were harvested at the indicated points and lased in RIPA buffer (150 mM NaCl, 1% SDS, 10 ug/ml leupeptin, 1 mM aprotinin, 50 mM Tris-Cl, pH 7.4) containing a protease inhibitor PMSF. After 3 freeze-thaw cycles in liquid nitrogen, the resulting cell lysates were cleared by a 15 min 12,000 × g centrifugation at 4°C and separated by 10% SDS–PAGE. After electrophoresis, the proteins were transferred to polyvinylidene difluoride-plus membranes. The blots were blocked with 50 g/L nonfat milk in PBST washing buffer (PBS, 0.05% tween-20) for 3 h and incubated with 1 : 1,000 diluted primary antibodies (LC3, p-Bcl-2, HMGB1, and a-actin) for 3 h, then stained with 1 : 2,000 diluted ALP-conjugated secondary antibodies for 1 h at room temperature. Bound antibodies were revealed by ECL (Supersignal West Pico Tial Kit) (PIERC).

Luciferase assay
COS-7 cells were co-transfected with JNK-luciferase or ERK-luciferase reporter plasmids in various combinations, having been transfected and cultured for 48 hours, the luciferase activity assay was performed in order to detect the HMGB1 gene in the signal path ways. Relative luciferase activity was normalized for transfection efficiency by co-transfection with pCMV-LacZ and spectrophotometric analysis.

Statistical analysis
Data are given as the mean SEM. Significance of differences between groups was determined by two-tailed Student’s t test or Fisher’s LSD test. P < 0.05 was considered significant.

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REFERENCES
Supplemental Fig. 1

Human Myeloid Leukemia Cells Autophagy

Supplemental Table 1

The specific oligonucleotide sequences of primers used in semi-Quantitative RT-PCR

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<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<td>HMGB1</td>
<td>5’-TTTCAAAACAAAGATGCCACA-3’</td>
<td>5’-GGTCCCTAATAACTCCTAAGCAGATA-3’</td>
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<tr>
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<td>5’-CTGGGCTGTGGAATGTAATG-3’</td>
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