Abstract. The aim of the present study was to investigate the effects of high-mobility group protein B1 (HMGB1) silencing on the susceptibility of retinoblastoma (RB) cells to chemotherapeutic drugs and the underlying molecular mechanisms. Western blot analysis revealed that vincristine (VCR), etoposide (ETO) and carboplatin (CBP) significantly increased the expression of HMGB1 in Weri-Rb-1 and Y79 cells compared with the untreated control (P<0.01). siRNA HMGB1 and siRNA negative control (NC) were transfected to Y79 cells by Lipofectamine™ 2000 and, following VCR treatment, the expression of HMGB1 and nuclear factor-κB (NF-κB) was analyzed. siRNA HMGB1 transfection silenced HMGB1 expression. The cytotoxicity of VCR to cells with and without siRNA HMGB1 was investigated by methyl thiazolyl tetrazolium (MTT) assay. siRNA HMGB1 markedly reduced the IC50 value of VCR to RB cells through downregulating the expression of NF-κB, similar to pyrrolidinedithiocarbamate (PDTC). Moreover, following siRNA HMGB1, siRNA NC and ammonium PDTC treatment, the apoptosis of RB cells with VCR incubation was evaluated by Hoechst staining, and the expression of cleaved caspase-3, cleaved poly(ADP-ribose) polymerase (PARP), Beclin 1 and p62 were determined with western blot analysis. The LC3 puncta were determined with immunofluorescence assay. The results demonstrated that VCR treatment significantly downregulated the expression of cleaved caspase-3, cleaved PARP and p62, and upregulated the expression of Beclin 1 in RB cells (P<0.01). Similar to the NF-κB inhibitor PDTC, siRNA HMGB1 significantly promoted apoptosis and suppressed autophagy of VCR-treated RB cells through reversing the effects of VCR on these signaling molecules (P<0.01). Therefore, HMGB1 silencing promoted the susceptibility of RB cells to chemotherapeutic drugs through downregulating NF-κB.

Introduction

Retinoblastoma (RB) is a common intraocular malignant tumor in infants that develops from embryonic retinal nervous tissue. RB frequently occurs in children aged ≤3 years, and several patients often succumb to the disease as a result of its high-grade malignancy and systemic metastases (1,2). At present, among the therapeutic schedules for RB, including photocoagulation, condensation, radiotherapy and chemotherapy, chemotherapy is the standard treatment. However, the presently available chemotherapeutics, such as vincristine (VCR), etoposide (ETO) and carboplatin (CBP), often result in drug resistance, which is associated with a low success rate or a high recurrence rate. Therefore, investigating the mechanism underlying the development of drug resistance in RB and overcoming this obstacle will greatly improve the survival rate and prognosis of the patients (3,4).

High-mobility group protein B1 (HMGB1) is a nuclear chromatin protein with strong mobility. HMGB1 is able to move freely and rapidly among various proteins and chromatin and selectively binds to specific sites. HMGB1 has been found to be closely associated with cancer drug resistance. When exposed to chemotherapeutics, cancer cells, such as osteosarcoma, colon cancer, endometrial cancer, lung cancer and leukemia cells, release higher amounts of HMGB1 and, subsequently, HMGB1 reduces the sensitivity of cancer cells to chemotherapeutics through enhancing autophagy and suppressing apoptosis (5-8).

The molecular mechanisms linked to HMGB1 have been previously investigated (9-12). HMGB1 affects cell behavior, including inflammation, proliferation, metastasis and invasion, by binding to the receptor for advanced glycation endproducts (RAGE), Toll-like receptor (TLR)-2, TLR-4 and TLR-9,
and then regulating downstream signaling pathways. Moreover, regardless of the type of receptor HMGB1 binds to, it affects cancer cell apoptosis and autophagy via finally transducing the signals to nuclear factor-κB (NF-κB) (9-12). Therefore, it was hypothesized that the HMGB1/NF-κB pathway may also play a crucial role in the drug resistance of RB cells. We herein performed a series of experiments, including gene silencing, cell viability, apoptosis, autophagy and western blot analysis, in order to confirm this hypothesis.

Materials and methods

Materials and cell culture. Ammonium pyrrolidinedithiocarbamate (PDTC), bicinechonic acid (BCA) kit, Hoechst staining kit, methyl thiazolyl tetrazolium (MTT), and horse-radish peroxidase-labeled goat anti-rabbit IgG (H+L; A0206) were purchased from Beyotime Institute of Biotechnology (Nantong, China). Lipofectamine™ 2000 was purchased from Invitrogen; Thermo Fisher Scientific (Carlsbad, CA, USA). Rabbit anti-Beclin 1 (ab62557), anti-p62 (ab56416), anti-cleaved caspase-3 (ab2302), anti-cleaved poly(ADP-ribose) polymerase (PARP; ab32057), anti-NF-κB (ab16502), anti-LC3 (ab48394), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ab8245) and anti-HMGB1 (ab79823) polyclonal antibodies were from Abcam (Cambridge, MA, USA). VCR, 5-ethyladenosine (EDA) (Sigma-Aldrich; Merck KGaA (St. Louis, MO, USA). siRNA HMGB1 (sense, 5'-CCC GUU AUGAAGAGAA CT3'-3' and antisense, 5'-AUUUCU CUUUAUACCGGGTT-3'), the negative control (siRNA NC, sense, 5'-UUCUCCGAACGUACGUTT-3' and antisense, 5'-ACGUGACGUUCAGAGATT-3'), and FAM-labeled siRNA were prepared by GenePharma (Shanghai, China). The human RB cell lines Weri-Rb-1 and Y79 were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM media containing 10% (v/v) fetal bovine serum (FBS) (both from HyClone, Logan, UT, USA).

HMGB1 levels after drug treatment. Weri-Rb-1 and Y79 cells were seeded into 6-well plates. After 48 h, 10 µM VCR, 5 µM ETO, and 1 µM CBP was added to each well. The cells were then incubated for 48 h, collected, and subjected to western blot analysis as described below.

Cells transfection. siRNA HMGB1, siRNA NC and FAM-labeled siRNA were transfected into cells using the Lipofectamine™ 2000 kit, according to the manufacture’s instructions. When the confluence of Y79 cells reached 50-70%, the mixture of siRNA and Lipofectamine™ 2000 was added to the cells. After 6 h, the medium was replaced by DMEM supplemented with 10% (v/v) FBS. After incubation for 48 h at 37°C and 5% CO2, FAM-labeled cells were photographed under a fluorescence microscope and the transfection efficiency was calculated by semi-quantitatively analyzing fluorescence intensity with Image-Pro Plus software. Other cells were submitted to western blot analysis for HMGB1 determination.

Cell viability. Y79 cells were seeded into 96-well plates and transfected with siRNA HMGB1 and siRNA NC, as described above. After 48 h, the cells were treated with 0.1, 0.3, 1, 3, 10, 30, 100 and 300 µM VCR. After a further 48 h, 20 µl MTT (5 mg/ml) was added to each well. After incubation for 4 h, the medium was discarded and 150 µl dimethyl sulfoxide was added to each well. After vibration for 10 min, the plates were subjected to absorbance determination at 560 nm using a microplate reader (Infinite F200/M200; Tecan, Mannedorf, Switzerland). Cells treated with VCR and without siRNA transfection served as control. The relative cell viability and the IC50 value of VCR to Y79 were calculated.

Cell apoptosis. Y79 cells were seeded into 96-well plates and were transfected with siRNA HMGB1 and siRNA NC, as described above. Additional cells were exposed to 10 µM PDTC. After incubation for 48 h, 10 µM VCR was added into each well. After a further 48 h, the cells were treated with the Hoechst staining kit according to the manufacturer's instructions. The cells were then visualized under a microscope (TS100; Nikon, Tokyo, Japan).

Western blot analysis. Y79 cells were seeded into 96-well plates and transfected with siRNA HMGB1 and siRNA NC, as described above. Additional cells were exposed to 10 µM PDTC. After incubation for 48 h, VCR was added to each well. After a further 48 h, the cells were collected and lysed in RIPA buffer for 30 min. The cell lysate was then centrifuged for 10 min at 7,000 g and 4°C. The supernatant was carefully collected and total protein concentration was analyzed with BCA kits. Subsequently, protein was denatured and loaded quantitatively to perform sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Membrane transfer was performed with the wet transfer method for 30-50 min. The membranes were incubated with primary antibody buffer (1:100; rabbit anti-HMGB1, anti-beclin 1, anti-p62, anti-cleaved caspase-3, anti-cleaved PARP, anti-NF-κB and anti-GAPDH polyclonal antibody) overnight at 4°C, followed by rinsing and incubation with secondary antibody buffer for 1-2 h at room temperature. After rinsing again, enhanced chemiluminescent (ECL) solution was dripped on the membrane and the membrane was exposed to a gel imaging system (Chemidoc™ XRS; Bio-Rad, Hercules, CA, USA). 'Quantity One’ software (Bio-Rad) was utilized to analyze the gray values of the protein strips.

Immunofluorescence assay. Y79 cells were seeded on glass slides and transfected with siRNA HMGB1 and siRNA NC, as described above. After incubation with PDTC and VCR, the cells were fixed with precooled 4% (w/v) paraformaldehyde for 10 min and rinsed with phosphate-buffered saline (PBS) three times, followed by incubation with serum containing 0.1% (w/v) Triton X-100 for 30 min and then with rabbit anti-LC3 polyclonal antibody at 4°C overnight. After washing with PBS, secondary antibody was added at room temperature for 1 h. The cells were again washed with PBS and stained with DAPI. Finally, the slides were mounted and observed under a confocal microscope. The average LC3 puncta were calculated by semi-quantitatively analyzing fluorescence intensity with Image-Pro Plus software based on at least 100 cells, which represents cell autophagy levels.

Statistical analysis. All data are expressed as mean ± standard deviation and were analyzed with Student’s t-test using
Results

Effects of chemotherapeutics on HMGB1 expression. As shown in Fig. 1, a faint blot representing a small quantity of HMGB1 was observed in the untreated control Weri-Rb-1 and Y79 cells. However, following VCR, ETO or CBP treatment, HMGB1 expression was found to be significantly upregulated in both types of RB cells (all P<0.01).

siRNA HMGB1 induces downregulation of HMGB1 expression in Y79 cells. After FAM-labeled siRNA HMGB1 transfection by Lipofectamine™ 2000, the percentage of positive Y79 cells reached 90%, suggesting successful transfection into RB cells (Fig. 2A). Western blot analysis revealed that, following siRNA HMGB1 transfection, the HMGB1
band was notably fainter compared with siRNA NC transfection (Fig. 2B). Quantitative results demonstrated that the gray value of HMGB1 in the siRNA HMGB1 group (0.09±0.01) was significantly smaller compared with that in the siRNA NC group (0.49±0.04; \( P<0.01 \)), indicating that siRNA HMGB1 induced downregulation of HMGB1 expression in RB cells.

**siRNA HMGB1 suppresses the proliferation of Y79 cells.** The effects of siRNA NC and siRNA HMGB1 on the inhibitory profile of VCR in Y79 cells was determined with the MTT assay (Fig. 3). Following VCR treatment at various concentrations for 48 h, the IC_{50} value of VCR in Y79 cells was 10.59±1.28. However, following siRNA NC and siRNA HMGB1 transfection, the IC_{50} values were 10.50±1.21 and 3.60±0.30, respectively, suggesting that siRNA HMGB1 enhanced the sensitivity of Y79 cells to VCR and reduced the effective concentration of VCR.

**siRNA HMGB1 promotes apoptosis of Y79 cells.** The apoptosis of Y79 cells was evaluated with Hoechst staining and the apoptotic cells were identified as light blue spots (Fig. 4), whereas non-apoptotic cells were not stained. Compared with siRNA NC, siRNA HMGB1 and PDTC observably promoted the apoptosis of Y79 cells following VCR treatment.

**siRNA HMGB1 and PDTC downregulate the expression of NF-κB.** As shown in Fig. 5, compared with the untreated control, the expression of NF-κB was markedly elevated in VCR-treated cells; the gray values in the control and VCR groups were 0.13±0.01 and 0.88±0.08, respectively (\( P<0.01 \)). When siRNA NC and siRNA HMGB1 were transfected into Y79 cells prior to VCR treatment, a dark band remained in the siRNA NC group, while the band in the siRNA HMGB1 group was almost invisible. The gray value in the siRNA NC group was significantly higher compared with that in the siRNA HMGB1 group (\( P<0.01 \)). Similarly, the expression of NF-κB was markedly inhibited by PDTC.

**siRNA HMGB1 and PDTC upregulate the expression of cleaved caspase-3 and cleaved PARP.** Qualitative and quantitative western blot analysis revealed that, after VCR treatment, the expression of cleaved caspase-3 and cleaved PARP in Y79 cells was significantly reduced (both \( P<0.01 \); Fig. 6). However, siRNA HMGB1 and PDTC treatment significantly increased the expressions in VCR-treated cells compared with siRNA NC (all \( P<0.01 \)), indicating that siRNA HMGB1 and PDTC were able to reverse the effects of VCR on cleaved caspase 3 and cleaved PARP expression in RB cells.

**siRNA HMGB1 and PDTC suppressed autophagy by regulating the expression of beclin 1 and p62.** The LC3 puncta in cells represented autophagy. As shown in Fig. 7A, the LC3 puncta were notably increased in VCR- and siRNA NC-treated cells. However, in the siRNA HMGB1 and PDTC groups, the LC3 puncta were significantly decreased (both \( P<0.01 \)), suggesting the effective inhibition of siRNA HMGB1 and PDTC on autophagy of Y79 cells. Furthermore, Y79 cells treated with VCR for 48 h produced a significantly higher amount of beclin 1 and lower amount of p62 compared with the control (both \( P<0.01 \); Fig. 7B). When cells were treated with siRNA NC, siRNA HMGB1 and PDTC prior to VCR treatment, cells in both the siRNA HMGB1 and PDTC groups released a significantly lower amount of beclin 1 and higher
amount of p62 compared with the siRNA NC group (all \( P<0.01 \)), suggesting that siRNA HMGB1 and PDTC were able to reverse the effects of VCR on beclin 1 and p62 expression in RB cells.

**Discussion**

HMG proteins were first identified by Goodwin in thymic cell in 1973 (13) and gained their name due to their high migration rates.
HMGB proteins are composed of classic HMG proteins and HMG motif proteins. We herein investigated the classic HMG proteins that are abundant in eucaryotes, namely HMGB, HMGA and HMGN, with HMGB being the most abundant. HMGB contains a special structural domain HMG-box and comprises three proteins, HMGB1, HMGB2 and HMGB3. The HMGB1 gene is located on chromosome 13q12 and its protein sequence is highly conserved, with two homologous HMG-boxes. HMGB1 has been found to affect the expression of downstream proteins, cell apoptosis, cell proliferation and autophagy through binding to endonuclear DNA and then regulating multiple gene transcriptions and gene recombination (14,15).

Moreover, the expression of HMGB1 was higher in tumors with poor differentiation and optic nerve invasion (16). In addition, numerous studies demonstrated that HMGB1 was associated with cancer resistance to chemotherapy. Luo et al reported that doxorubicin promoted the expression of HMGB1 in CT27 colon cancer cells (9). Liu et al also demonstrated that chemotherapeutic drugs upregulated the expression of HMGB1 in leukemia cells (17). Zhang et al reported that doxorubicin, cisplatin and methotrexate stimulated the upregulation of HMGB1 expression in A549 lung cancer cells, and the expression of HMGB1 in A549/DDP drug-resistant cells was significantly higher compared with that in A549 cells (18). Therefore, in the present study, the expression of HMGB1 in the RB cell lines Weri-Rb-1 and Y79 after treatment with 10 µM VCR, 5 µM ETO and 1 µM CBP for 48 h was investigated through western blot analysis, and the results demonstrated that all three drugs markedly elevated the expression of HMGB1 in RB cells. VCR and Y79 cells were employed as the model drug and model RB cells in the following experiments, respectively.
In addition, shRNA HMGB1 reduced the IC_{50} value of cisplatin to A549/DDP cells and enhanced the susceptibility of A549/DDP cells to cisplatin (18). HMGB1-neutralizing antibody may improve the sensitivity of leukemia cells to chemotherapeutic drugs (17). Similarly, HMGB1 gene expression was silenced in Y79 cells by siRNA HMGB1 transfection. Based on the cell viability analysis by MTT assay, it was observed that siRNA HMGB1 significantly reduced the IC_{50} value of VCR to RB cells and enhanced the inhibitory effect of VCR on RB cells. These results suggested that HMGB1 was closely associated with the chemotherapeutics of RB cells and the downregulation of HMGB1 expression was able to notably intensify the susceptibility of RB cells to chemotherapeutic drugs.

Due to having two homologous box structural domains, the HMGB1 gene can bind to the corresponding receptors RAGE, TLR-2 and TLR-4. These binding complexes then activate multiple signaling pathways, including NF-κB, mitogen-activated protein kinase, phosphatidylinositol 3-kinase/AKT, and Janus kinase/signal transducer and activator of transcription, consequently regulating cell proliferation, differentiation and autophagy. Among those pathways, NF-κB was the most common pathway of HMGB1 receptors RAGE and TLR-4, and HMGB1 significantly affected cell behavior via activating the NF-κB pathway (9-12). Following activation by upstream signals, NF-κB with special DNA sequences shifts into the nucleus, binds to promoter or enhancer of downstream target genes, and then regulates the expression of apoptosis-related genes and inflammatory factors. In addition, NF-κB was reported to be involved in the chemotherapy of several types of cancer (19,20). Therefore, in the present study, the expression of NF-κB in Y79 cells treated by VCR for 48 h was evaluated through western blot analysis and it was revealed that, although VCR treatment obviously elevated the expression of NF-κB, after siRNA HMGB1 transfection and PDTC (specific NF-κB inhibitor) treatment, the expression of NF-κB was markedly downregulated. These findings suggest that siRNA HMGB1 promoted death and apoptosis of RB cells through markedly decreasing the high level of NF-κB induced by chemotherapeutic drugs.

Multiple factors and processes are involved in the resistance of cancer cells to chemotherapeutic drugs, mainly including membrane glycoprotein-mediated drug efflux, DNA repair and apoptosis pathway abnormalities. The inhibition of cancer cell apoptosis is a major cause of cancer drug resistance and chemotherapy failure; thus, it was hypothesized that the induction of cancer cell apoptosis may effectively enhance the sensitivity of cancer cells to chemotherapeutic drugs and suppress the development of cancer drug resistance (21,22). Therefore, in the present study, the apoptosis of Y79 cells following treatment with siRNA HMGB1, PDTC (a specific NF-κB inhibitor) and VCR was investigated. The results of Hoechst staining revealed that both siRNA HMGB1 and NF-κB inhibitor obviously aggravated the apoptosis of RB cells induced by chemotherapy. In addition, cleaved caspase-3 and cleaved PARP are markers of cell apoptosis, whereas caspase is the executor of cell apoptosis. As it is in the middle and downstream of the caspase cascade reactions, caspase-3 is the principal effector molecule of the apoptotic process and the intersection point of the apoptosis pathway. As a DNA repairase in eukaryocytes, PARP is the substrate of the caspase family. The cleavage of PARP reflects the activation of the caspase family. In the present study, the expression of cleaved caspase-3 and cleaved PARP in Y79 cells after treatment with siRNA HMGB1, PDTC and VCR was determined; the results of qualitative and quantitative western blot analysis demonstrated that siRNA HMGB1 and PDTC markedly upregulated their expression in RB cells after chemotherapy. This result was in agreement with previous studies (18, 23). Zhang et al revealed that shRNA HMGB1 promoted cell apoptosis after cisplatin induction through upregulating the expression of cleaved caspase-3 (18). Liu et al demonstrated that the downregulation of HMGB1 in chemotherapeutic drug-treated Y79 cells may induce oxidative stress injury and DNA damage, increase the activity of caspase-3, and upregulate the expression of cleaved caspase-3 and cleaved PARP. Accordingly, it was indicated that siRNA HMGB1 intensified chemotherapy-induced apoptosis in RB cells and promoted the susceptibility of RB cells to chemotherapeutic drugs through downregulating the expression of NF-κB and subsequently upregulating the expression of cleaved caspase-3 and cleaved PARP.

Furthermore, HMGB1 may alleviate the resistance of cancer cells to drugs by restraining autophagy and facilitating cell apoptosis (17,18). As a self-rescue mode under conditions of stress or alimentary deficiency, autophagy not only eliminates aging or damaged organelles, but also repairs DNA damage and protein misfolding. The chemotherapeutic drug resistance in a number of cancer cells was enhanced along with increased activity of autophagy; thus, the development of drug resistance in cancer cells may be inhibited and the sensitivity of cancer cells to drugs may be increased through suppressing the increase in cancer cell autophagy (24). In the present study, the autophagy protein markers beclin 1 and p62 in VCR-treated Y79 cells were determined after siRNA HMGB1 and PDTC treatment using western blot analysis. It was observed that, after HMGB1 gene silencing by siRNA or NF-κB inhibition by PDTC, beclin 1 expression was downregulated and p62 expression was upregulated. It was suggested that the chemotherapeutic drug-induced autophagy in RB cells was suppressed by regulating beclin 1 and p62. Similarly, exogenous HMGB1 reduced the sensitivity of leukemia cells to chemotherapeutic drugs and enhanced cell autophagy (17). HMGB1 silencing by shRNA may promote cell apoptosis induced by cisplatin and inhibit the expression of autophagy-related proteins (18).

In conclusion, VCR, ETO and CBP markedly upregulated the expression of HMGB1 in RB cells. However, after HMGB1 silencing by siRNA, the susceptibility of RB cells to chemotherapeutic drugs and the apoptosis of RB cells with chemotherapeutic drug treatment were notably improved. In addition, siRNA HMGB1 inhibited the expression of NF-κB and, similar to the NF-κB inhibitor PDTC, siRNA HMGB1 treatment significantly increased the expression of cleaved caspase-3, cleaved PARP and p62, and reduced the expression of beclin 1. Consequently, siRNA HMGB1 promoted apoptosis and suppressed autophagy of RB cells through downregulating NF-κB. Therefore, the downregulation of the HMGB1/NF-κB pathway was able to overcome the resistance of RB cells to chemotherapy. These results may contribute to the molecular diagnosis and targeted therapy of RB based on HMGB1.
References


