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Exogenous High-Mobility Group Box 1 Inhibits Apoptosis and Promotes the Proliferation of Lewis Cells via RAGE/TLR4-Dependent Signal Pathways

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Abstract

Upregulated high-mobility group box 1 (HMGB1) has been found in many diseases. Nevertheless, the function of HMGB1 on modulating the proliferation of lung cancer cells (Lewis cells) and inhibiting apoptosis is poorly understood, as well as the involved intracellular signalling. In the present study, we firstly found the apoptosis of Lewis was increased following Hanks' balanced salt solution (HBSS)-induced starvation, while it was rescued after exogenous HMGB1 protein was added; furthermore, the receptor for advanced glycation end products (RAGE) and Toll-like receptor (TLR4) could coordinately improve the proliferation of tumour cells *in vitro,* and HMGB1 could enhance the phosphorylation of PI3K/Akt and Erk1/2, inhibit the expression of pro-apoptosis protein Bax and promote the expression of anti-apoptosis protein Bcl-2. These findings clearly demonstrated that HMGB1–RAGE/TLR4- PI3K-Akt/Erk1/2 pathway contributed to the proliferation of Lewis. Moreover, our observations provide experimental and theoretical basis for clinical biological therapy for cancers; it also may be a new target for intervention and treatment of lung cancer.

Introduction

High-mobility group box-1 (HMGB1), originally described as a nuclear non-histone protein with DNA-binding domains, has been implicated as an important extracellular danger signalling molecule and a potent pro-inflammatory cytokine extracellular damage-associated molecular pattern (DAMP) [1]; it overexpressed in many tumour cells and involved in the pathogenesis of many diseases, such as sepsis, acute pancreatitis, rheumatoid arthritis and pneumonia [2]. HMGB1 can trigger inflammation, lead to cell migration, proliferation and tumour metastasis following release into the extracellular space, promote autophagy, activate innate immune responses and act as a chemoattractant for endothelial cells, smooth muscle cells and fibroblasts [3, 4]. However, specific extracellular and intracellular signals that regulate the proliferation of lung cancer cells (Lewis) and apoptosis are poorly understood. Therefore, the mechanism of HMGB1 function and clinical application value need to be further studied.

Several cell surface receptors are implicated in HMGB1 signalling, including receptors for advanced glycation end products (RAGE), Toll-like receptor family (such as TLR-2, 4, 9) and most recently were joined by T cell immunoglobulin and mucin domain 3 (TIM-3) [5]. RAGE is a multiligand-binding member of the immunoglobulin super-family. The full-length receptor consists of an extracellular region formed from 1V-type immunoglobulin domain needed for ligand binding and 2 C-type immunoglobulin domains; these domains are followed by a single, short transmembrane domain and a short cytoplasmic domain that is essential for RAGE-mediated signal transduction [6]. Meanwhile, RAGE is expressed in a wide variety of tissues, including the lung. More and more data indicated that HMGB1 combine with RAGE or TLRs can lead to cellular activation and thus prolonged inflammation, proliferation and apoptosis [7, 8]. The ligation of HMGB1 to RAGE or TLRs results in the activation of diverse intracellular signalling pathways including phosphoinositide 3-kinase (PI3K) and its downstream serine/threonine kinase (Akt) and mitogen-activated protein kinase (MAPK, Erk1/2) [9], whose activation is believed to play a major role in regulating the activation and proliferation of Lewis.

SCANDINAVIAN JOURNAL OF Immunology AN INTERNATIONAL MONTHLY JOURNAL

Based on these findings, the purpose of this study is to investigate whether HMGB1 can induce proliferation of Lewis and inhibit apoptosis and whether RAGE or TLRs-dependent signal pathway is involved in the mechanism. Our results demonstrated that apoptosis was enhanced during Hanks' balanced salt solution (HBSS) starvation, but HMGB1 protein could attenuate HBSS-induced Lewis apoptosis. Furthermore, HMGB1 could significantly stimulate proliferation of Lewis and inhibit apoptosis *in vitro*, and RAGE/TLR4-dependent PI3K/Akt and Erk1/2 signal pathways are involved in the HMGB1 induced proliferation of Lewis. To our knowledge, this is the first report on HMGB1-associated Lewis proliferation and apoptosis. Thus, the present data were to provide evidence and a potential theoretical basis for HMGB1 regulation of lung cancer cells proliferation and apoptosis to further elucidate the molecular mechanism of HMGB1 involvement in various pathologic conditions that can affect the lung. These data further indicate a significant function of HMGB1 and its possibility of being an effective target to treat lung cancer.

Materials and methods

Cell culture and apoptosis assay. Lewis cells were cultured in DMEM medium (Gibco, Grand Island, NY, USA), supplemented with 10% heat-inactivated calf serum (CS), 100 U/ml penicillin and 100 mg/l of streptomycin and maintained at 37 °C under 5% CO_2 atmosphere.

The apoptotic ratios of Lewis cells were determined with the Annexin V-FITC/PI apoptosis detection kit (Invitrogen, Shanghai, China). Briefly, after 0, 4, 8, 12 h HBSS and 1 μg/ml, 10 μg/ml HMGB1 protein (Catalog Number H4652; Sigma-Aldrich, Shanghai, China) treatment, the cells were collected and washed twice with ice-cold PBS buffer, resuspended in 100 μl of 1× Annexin-binding buffer, incubated with 5 μl of Annexin V conjugated to FITC and 1 μl PI (100 mg/ml) for 15 min at room temperature, and then resuspended in 400 μl of 1× Annexin-binding buffer and analysed by flow cytometry. Apoptotic morphological changes in the nuclear chromatin of cells were detected by Hoechst 33342 staining. Lewis cells were seeded in the six-well plates. After overnight growth, cells were washed with phosphate-buffered saline (PBS) 5 min for five times and then treated with 0.5% Triton 100 (dissolved in 1% BSA). After 30 min, cells were washed with PBS 5 min for five times and then incubated with Hoechst 33342 staining solution for 10 min. After three washes with PBS, the cells were viewed under a fluorescence microscope.

Cell proliferation assay by MTT and CFSE. Cells were plated in 96-well plates (5000 per well) in 10% CS-DMEM overnight to allow attachment. After the plate was washed three times with PBS to remove suspended cells, 0.1 ml HBSS and 10 μg/ml HMGB1 were added and cultured for 6 h at 37 °C, 5% CO_2, and then the plate was washed and cultured for another 4 h in DMEM medium with 5 mg/ml methyl thiazolyl-diphenyl-tetrazolium bromide (MTT; AMRESCO, USA) solution 10 μl. The optical density was read at 490 nm following DMSO (Sinopharm Chemical Reagent Co., Shanghai, China) was added. Before the data were analysed, the cells counts were converted to percentages relative to the control group, which was regarded as 100%.

Lewis cells were collected and resuspended in 1 ml PBS with 10 μM CFSE [5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester, CFDASE; Invitrogen] 1 ml at 37 °C for 10 min, which were protected from light and blending, then followed by added 5 ml DMEM to stop reaction. Then, cells were washed twice with PBS, and cells were plated in six-well plates with two groups of different treatments for 48 h. After 2 days, cells were detected and analysed by flow cytometry.

Polymerase chain reaction (PCR) assay. Total RNA was extracted using Trizol (Invitrogen). According to the manufacturer's instructions, reverse transcription was obtained by PrimeScript RT reagent kit (Takara, Dalian, China) and PCR conducted with a reverse transcription polymerase chain reaction Kit (Takara) using the following conditions: predegenerated 5 min at 94 °C, followed by a total of 35 cycles (30 s at 94 °C, 30 s at 60 °C and 1 min at 72 °C) and then fully extended 10 min at 72 °C. The primer sequences are given in Table 1.

Table 1 Primers used in PCR.

Primer	Oligonucleotide sequence (5′–3′)	Amplicon size (bp)	Annealing temperature (°C)
β-actin	GTGCCCATCTACGAGG GCCACAGGATTCCATAC	333	60
RAGE	TCCCGATGGCAAAGAAACAC CAGAGATGGCACAGGTCAAGGT	290	62
TLR-2	CAGACGTAGTGAGCGAGCTG GGCATCGGATGAAAAGTGTT	389	60–65
TLR-4	TTCACCTCTGCCTTCACTACA GGGACTTCTCAACCTTCTCAA	227	60
TLR-9	GAAAGCATCAACCACACCAA ACAAGTCCACAAAGCGAAGG	304	60–65

Western blot. Lewis cells were collected. The cell lysates were separated by 1× SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membrane. PVDF was blocked with 5% non-fat milk for 4 h followed by incubation with the primary antibodies in TBST overnight at 4 °C with gentle shaking: the specific primary antibodies against phospho-PI3K kinase p85 (Tyr458)/p55 (Tyr199) [Cell Signaling Technology (CST), Shanghai, China], phospho-Akt (ser473) (CST), Bax (Proteintech, Chicago, IL, USA), Bcl-2 (Bioworld, Nanjing, China), phospho-p44/42 MAPK (Erk1/2, Thr202/Tyr204) (CST) and β-actin (Abcam, Cambridge, UK). After washing, the second antibody HRP-conjugated goat anti-mouse IgG (BOSTER, Wuhan, China) and goat anti-rabbit IgG (Abmart, Shanghai, China) for 1 h at room temperature. The ratio of each protein to β-actin was calculated as the relative quantification.

Immunofluorescence and flow cytometry analyse membrane TLR4 and RAGE expression. Lewis cells were seeded in the 24-well plates. After overnight growth, cells were

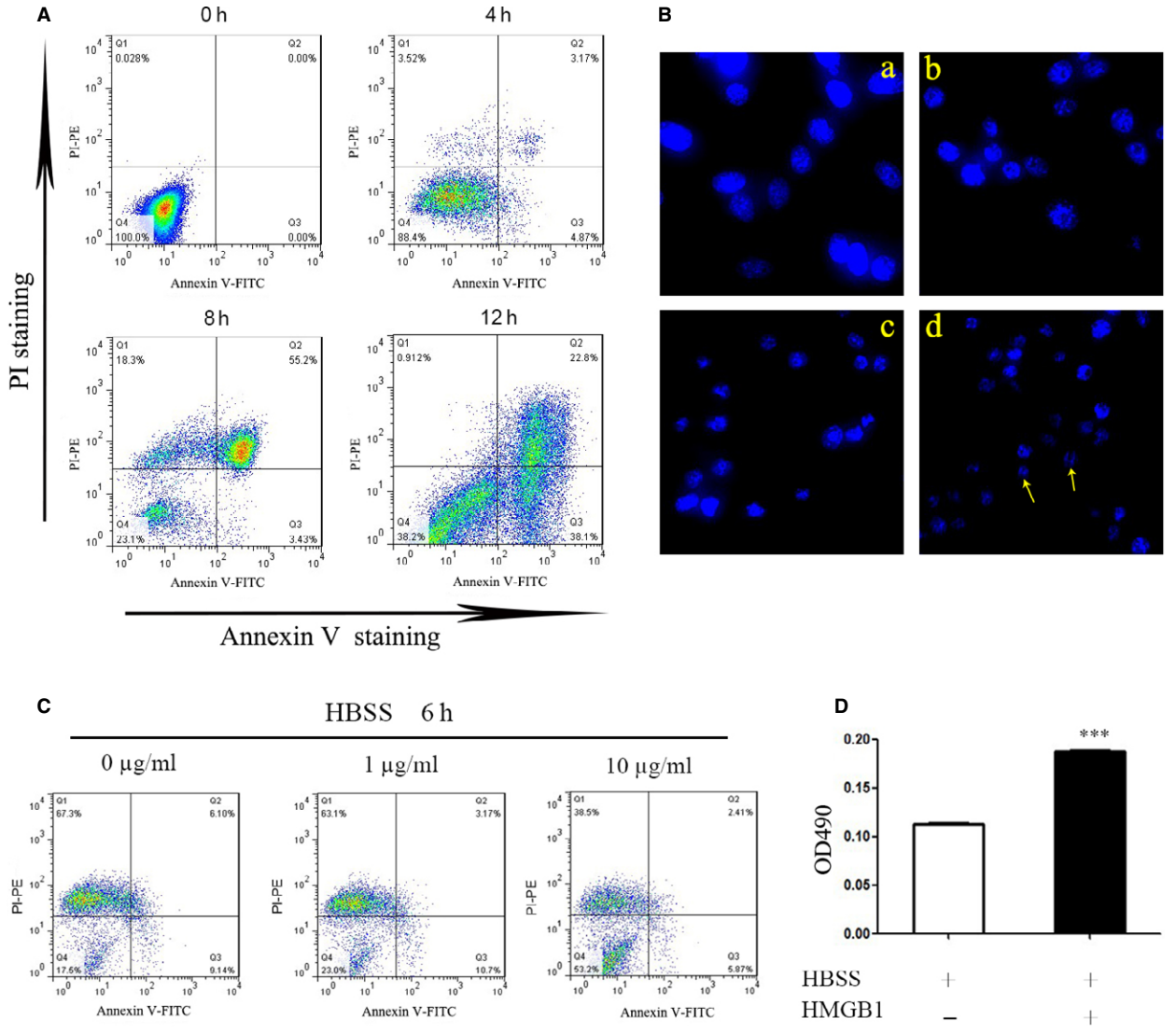

Figure 1 Hanks' balanced salt solution (HBSS) starvation induced Lewis apoptosis in a time-dependent manner and the exogenous HMGB1 protein rescued apoptosis. (A) The treated cells were fixed with HBSS for 0, 4, 8 and 12 h, stained with Annexin V-FITC/propidium iodide (PI) and analysed by flow cytometry. (B) Hochest 33342 staining was performed using fluorescence microscopy (×400). The image above shows Lewis cells treated with the HBSS for 0, 4, 8 and 12 h. (a) In controls, the majority of cells had uniformly stained nuclei. (b) Lewis treated with HBSS for 4 h showed morphological changes typical of apoptosis (i.e. nuclei fragmentation with condensed chromatin). (c) Cells treated with HBSS for 8 h, and the apoptosis cells have been clearly increased. (d) Arrows indicate cells treated with HBSS for 12 h, and the apoptosis cells had obvious morphological change (i.e. nuclei fragmentation with condensed chromatin), and the percentage of apoptosis was enhanced clearly. (C) 1 and 10 μg/ml HMGB1 protein was added to the upper transwell chamber containing the cells (HBSS stimulation for 6 h). (D) After treated with 10 μg/ml exogenous HMGB1 protein for 48 h, HMGB1 increased the survival percentage of Lewis and decreased the apoptosis rate of Lewis using MTT assay. Data were obtained from three independent experiments; $P < 0.05$ was considered statistically significant. ***$P < 0.005$.

washed five times with PBS for 5 min and then treated with 0.5% Triton 100 (dissolved in 1% BSA) for 30 min. Next, the cells were incubated with Anti-RAGE mAb (Abcam) or Anti-TLR4 mAb (Abcam) for 1 h, and PBS washed five times for 5 min. FITC-conjugated goat anti-rabbit IgG was added for 1 h, and PBS washed five times for 5 min. After that, Lewis cells were incubated with Hoechst 33342 (Sigma-Aldrich) staining solution for 10 min and viewed by fluorescence microscope.

Cells were plated in six-well plates overnight to allow attachment. Then, 10 μg/ml HMGB1 protein was added and cultured for 48 h. The cells were collected, and 1.5 μl 0.6 mg/ml RAGE antibody or 1 μl 1 mg/ml TLR4 antibody was added, washed with PBS and then 1 μl of FITC-rabbit IgG (Santa Cruz, Inc., Santa Cruz, CA, USA) with 100 μl of PBS was added, and finally the cells were analysed by flow cytometry.

Statistical analysis. Values are presented as the mean $\pm$ standard deviation (SD), in triplicate. Statistical analyses were performed using the Student's two-tailed unpaired *t*-test or one-way ANOVA analysis followed by Pearson's rank correlation, as appropriate. $P < 0.05$ was considered statistical significance.

Results

The exogenous HMGB1 protein rescues Lewis apoptosis in HBSS-induced starvation

Hanks' balanced salt solution starvation could significantly induce Lewis apoptosis in a time-dependent manner (Fig. 1A). Immunofluorescence analysis also showed an apoptosis morphological change (Fig. 1B). To address the effect of HMGB1 on HBSS-induced starvation, the exogenous HMGB1 protein was added in different concentrations, and the result showed that the survival percentage of Lewis cells was increased, but apoptosis was inhibited (Fig. 1C,D). The data indicated that the effect of HBSS starvation was reversed after Lewis cells treated by 10 μg/ml recombinant HMGB1.

HMGB1 inhibited apoptosis and promoted the proliferation of Lewis cells through RAGE/TLR4 signalling

As shown in Fig. 2, HMGB1 inhibited the Lewis cells apoptosis by decreasing the pro-apoptosis protein Bax expression and increasing anti-apoptosis protein Bcl-2 expression in a time-dependent manner.

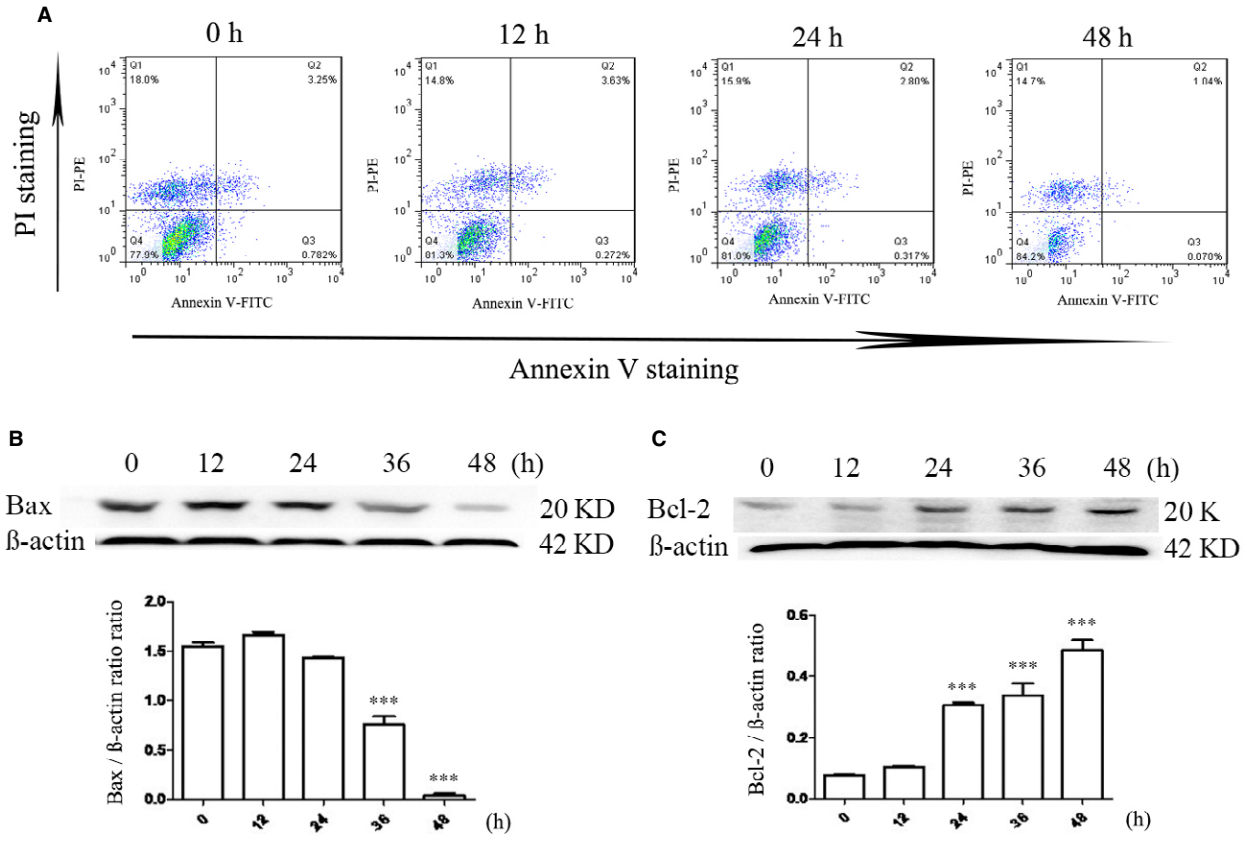

Figure 2 HMGB1 inhibited Lewis cells apoptosis. (A) 10 μg/ml exogenous HMGB1 protein was added to Lewis for 0, 12, 24 and 48 h. (B,C) After pretreated with HMGB1 (10 μg/ml) for 0, 12, 24, 36 and 48 h, Western blot detected the Bax and Bcl-2. Relative quantification of Bax and Bcl-2 protein was presented. Data were obtained from three independent experiments. $P < 0.05$ was considered statistically significant. ***$P < 0.005$.

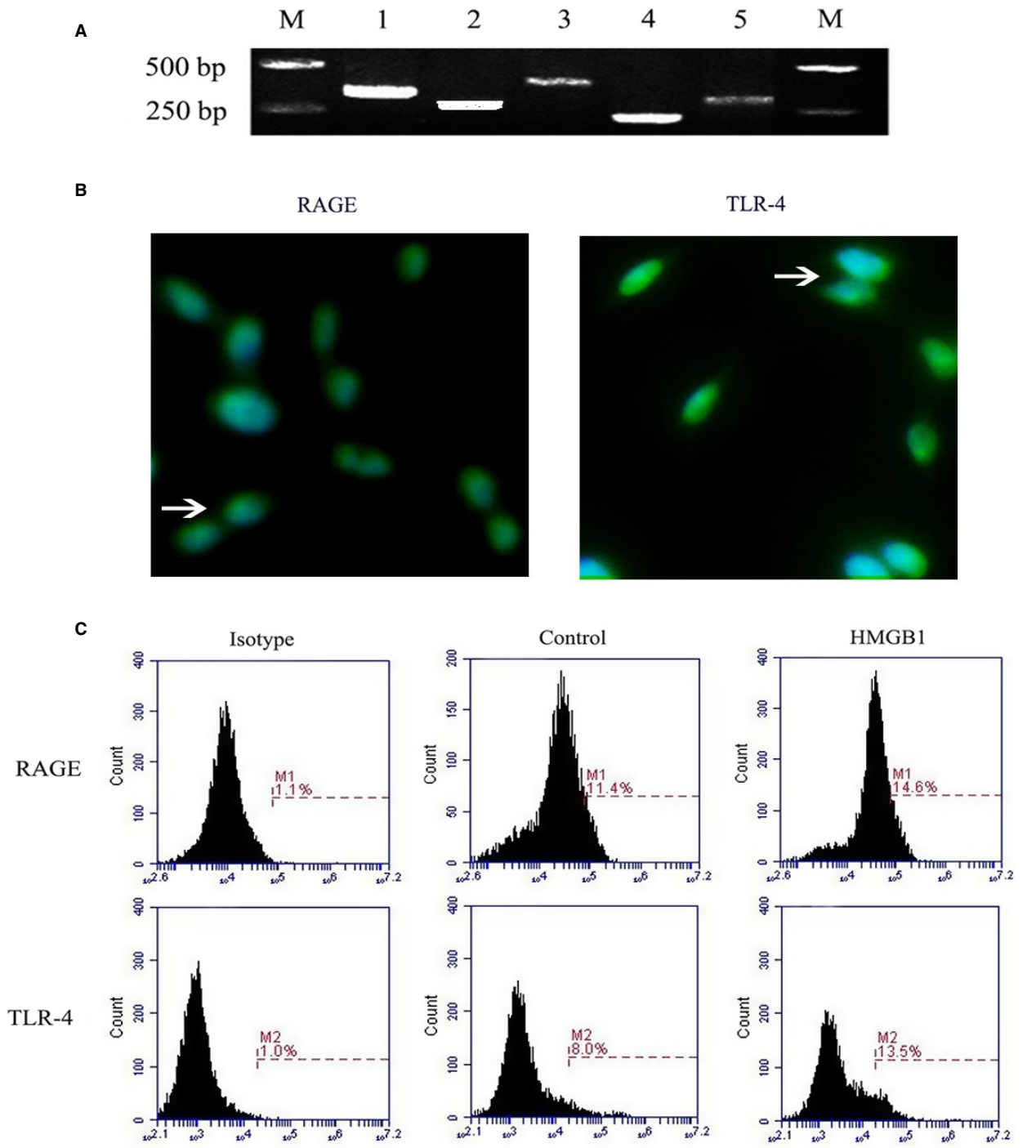

Figure 3 The expression of receptor for advanced glycation end products (RAGE) and TLR4 in Lewis cells. (A) PCR was used to detect relevant receptors of HMGB1 protein. M: Marker DL2000; 1: β- actin (333 bp); 2: RAGE (290 bp); 3: TLR2 (389p); 4: TLR4 (227 bp); 5:TLR9 (304 bp). (B) Hochest 33342 stains (blue fluorescence), 0.6 mg/ml RAGE antibody 1.5 μl, 1 mg/ml TLR4 antibody 1 μl and FITC-conjugated goat anti-rabbit IgG (green fluorescence) were performed using fluorescence microscopy (×400). (C) Compared to isotype and control groups, the expression of RAGE and TLR4 was significant increased after treatment of HMGB1 (10 μg/ml) for 48 h by flow cytometry. Data were obtained from three independent experiments.

Then, we wanted to know whether exogenous HMGB1 contributed to the proliferation of Lewis. Therefore, the potential receptors expressions of HMGB1 were detected on Lewis. Our PCR results showed that RAGE, TLR2, TLR4 and TLR9 were expressed on Lewis (Fig. 3A); RAGE and TLR4 expression were associated with cells proliferation. So RAGE and TLR4 expression were furthering confirmed on the surface of Lewis cell by

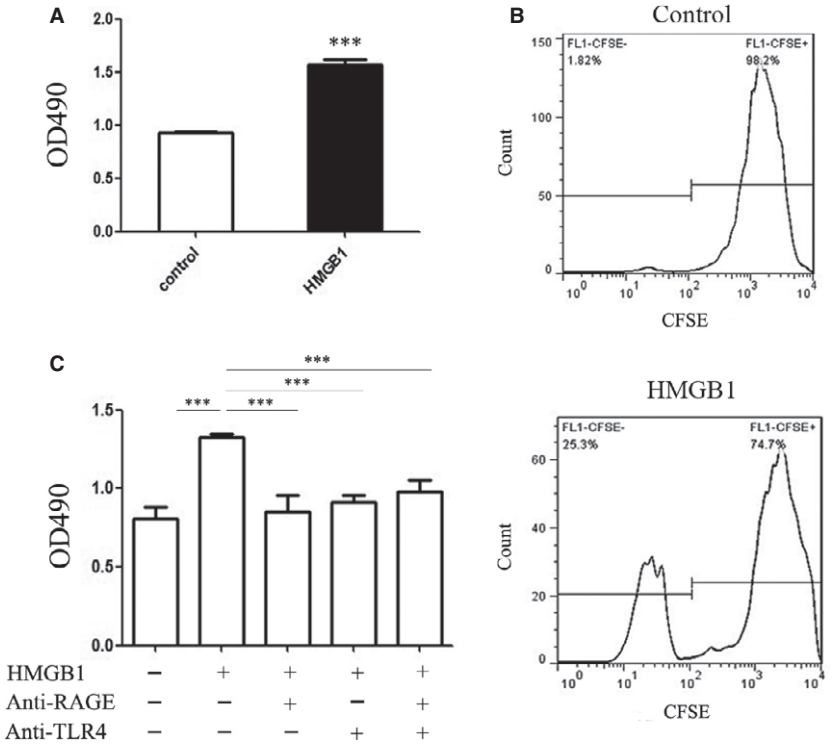

Figure 4 HMGB1 protein promoted proliferation of Lewis cells. (A) After treated with 10 μg/ml exogenous HMGB1 protein for 48 h, the number of Lewis was detected using MTT assay. Data were expressed as mean ± SD. (B) CFSE levels in untreated Lewis are low, and CFSE levels in Lewis treated with 10 μg/ml HMGB1 for 48 h were high. (C) Anti-RAGE mAb and Anti-TLR4 mAb (100 μg/ml) were added in Lewis cells within an hour prior and then cells were treated with HMGB1 protein (10 μg/ml) for 48 h and detected by MTT assay. Data were expressed as mean ± SD. Data were obtained from three independent experiments. $P < 0.05$ was considered statistically significant. CFSE, (5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester; RAGE, receptor for advanced glycation end products. ***$P < 0.005$

fluorescence microscope (Fig. 3B). FCM analysis was clearly demonstrated that RAGE and TLR4 expressions were inducible increase on Lewis treated by 10 μg/ml HMGB1 for 48 h (Fig. 3C). The Lewis cells were proliferation after the exogenous HMGB1 treatment and the apoptosis were ameliorated (Fig. 4A), and the FCM analysis was furthering confirmed the data that the untreated cells were $CFSE^{low}$ and treated cells were $CFSE^{high}$ (Fig. 4B).

To address whether RAGE and TLR4 were also involved in the HMGB1 mediating Lewis proliferation, the antibodies were employed to block RAGE and TLR4 on Lewis cells, respectively. As shown in Fig. 4C, after blockade, the Lewis cells proliferation was inhibited.

HMGB1 induced the activation of PI3K/Akt, Erk in Lewis cells

Challenge of Lewis with HMGB1 resulted in a transient increase in phosphorylation of PI3K, Akt and Erk1/2 within Lewis (Fig. 5A,B). To furthering confirm Erk1/2 signalling was involved in HMGB1-induced Lewis proliferation, Lewis pretreated with Erk1/2 inhibitors U-0126 (10 μM) for 1 h and then treated with 10 μg/ml HMGB1. The proliferation of Lewis was obviously decreased and compared with unpretreated group (*$P < 0.05$) Fig. 5(C,D).

Discussion

Under HBSS starvation, the intrinsic pathway of apoptosis (mitochondrial apoptosis) is activated. Starvation causes aberrant ROS generation and trigger oxidative signalling pathways of apoptosis, which involved hyperoxidation of DNA, lipids and proteins damage [1]. Numerous members of the caspase family have been identified, and it appears as if caspase-3 plays an important role [10, 11]. The Bcl-2 oncoprotein has been shown to act at the level or upstream of caspase-3 family activation to inhibit apoptosis induced by various stimuli and Bax, a pro-apoptosis protein, can activate the anti-apoptotic Bcl-2 family member [12]. In this study, we had investigated whether HBSS starvation induced Lewis apoptosis. HMGB1, as the prototypic DAMPs, could be released by apoptosis/necrosis cells [13] or actively secreted by monocytes/macrophages at sites of inflammation and/or tissue damage [14, 15]. The extracellular HMGB1 was capable of activating other cells involved in immune response and inflammatory reactions. HMGB1 had been reported to play important roles in promoting both cell survival and death by regulating multiple signalling pathways, including inflammation, proliferation, metabolism, apoptosis, autophagy, immunity, genome stability and metastasis.

In the present work, we found that the exogenous HMGB1 could rescue Lewis apoptosis under HBSS and promote the proliferation of Lewis (Fig. 1). However, there were two completely opposite conclusions about HMGB1 contributing to apoptosis or proliferation. Some researchers [14, 16–18] thought that HMGB1 could inhibit apoptosis by activating NF-κB, leading to overexpression of anti-apoptosis gene NF-κB, upregulating the inhibitor of apoptosis protein C-IAP2 and inhibiting caspase-3 and

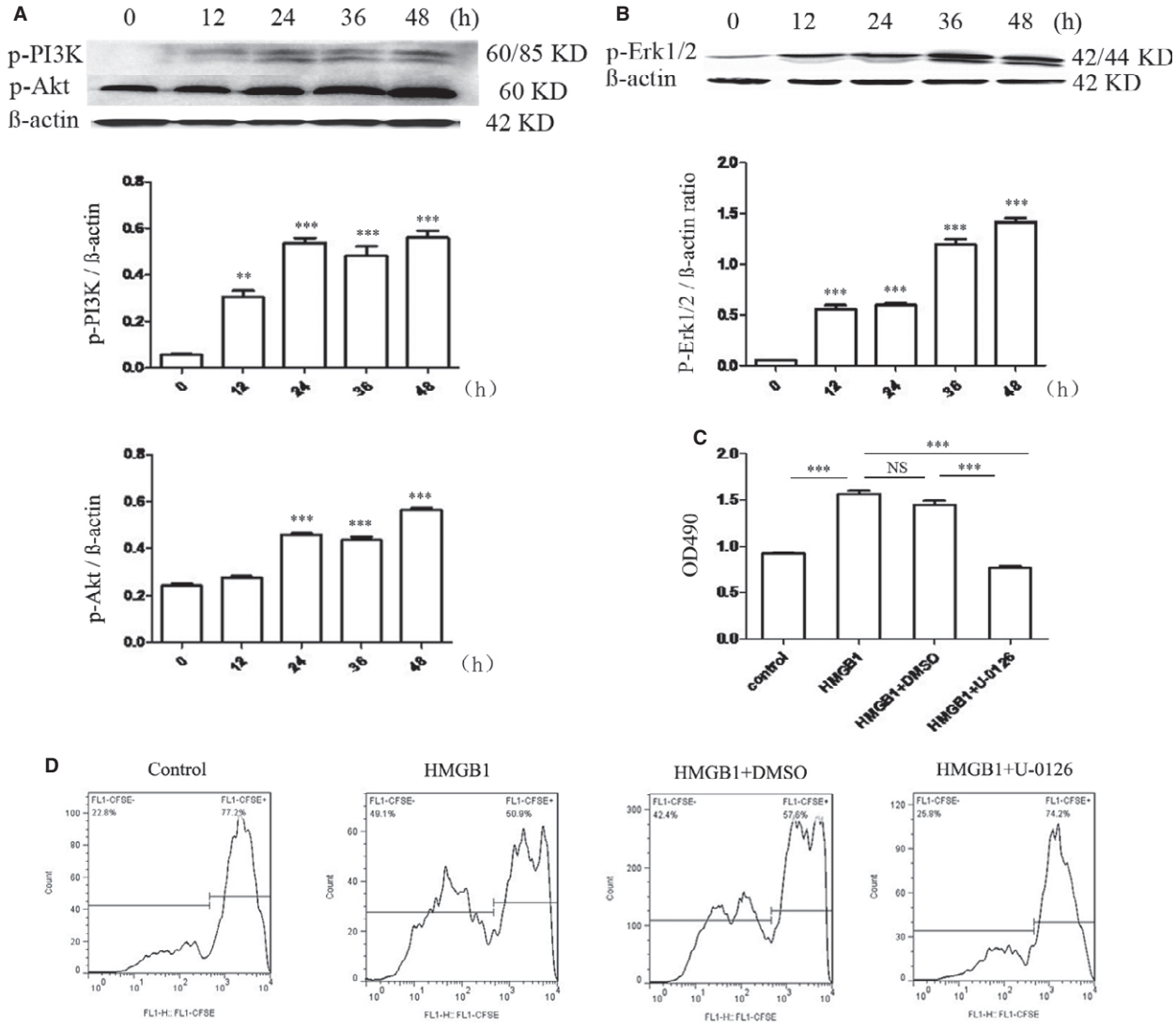

Figure 5 HMGB1 induced activation of PI3K, Akt and Erk1/2. Erk1/2 was involved in HMGB1-induced Lewis proliferation. (A) Representative of significantly elevated expressions of both p-PI3K and p-Akt in response to 10 μg/ml HMGB1 stimulation for 0, 12, 24, 36 and 48 h. Relative quantification of the active PI3K and Akt proteins was presented. *$P < 0.05$ compared with 0 μg/ml HMGB1 protein. (B) Lewis cells were stimulated by 10 μg/ml HMGB1 for 0, 12, 24, 36 and 48 h to be analysed the protein levels of phospho-Erk1/2 by Western blot. Compared to 0 μg/ml HMGB1 stimulation, the expression of p-Erk1/2 was markedly increased. Relative quantification of p-Erk1/2 protein was presented. *$P < 0.05$ compared with 0 μg/ml HMGB1. (C) The proliferation of Lewis pretreated with U-0126 (Erk1/2 inhibitor) for 1 h was analysed after their incubations with HMGB1 for 48 h by MTT assay. *$P < 0.05$ compared with the HMGB1 group. NS, not significant. (D) The proliferation of Lewis was analysed by flow cytometry and CFSE represented the Lewis proliferation as shown in the bottom panel. Data were obtained from three independent experiments. CFSE, (5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester. **$P < 0.01$; ***$P < 0.005$.

caspase-9 activity; which indicated that HMGB1 may be an anti-apoptotic protein [16, 19]. This conclusion was consistent with our data (Fig. 2). However, Geum-Youn Gwak *et al.* [20] reported that glycyrrhizin attenuates HMGB1-induced hepatocyte apoptosis by inhibiting the p38-dependent mitochondrial pathway. Ding *et al.* [21] also reported the HMGB1-TLR4 axis contributes to myocardial ischaemia/reperfusion injury via regulation of cardiomyocyte apoptosis, which were associated with the redox/oxide remodelling on C106, C23, C45 or other points of HMGB1. In future work, we should pay more attention to question. Above all, they considered HMGB1 protein could induce apoptosis in the present work.

It was reported that HMGB1 ligation could promote the cells' proliferation by activating the PI3K/Akt signalling pathway [22] or the MAPK pathway, such as p38, JNK and Erk1/2 [23, 24]. Our findings suggested that exogenous HMGB1 binding with receptor RAGE, TLR2/4 expressed on Lewis cells (Fig. 3), activated PI3K, Akt, Erk1/2 and contributed to the Lewis proliferation (Figs. 4 and 5).

In conclusion, our results clearly demonstrated that exogenous HMGB1 could rescue apoptosis of Lewis with

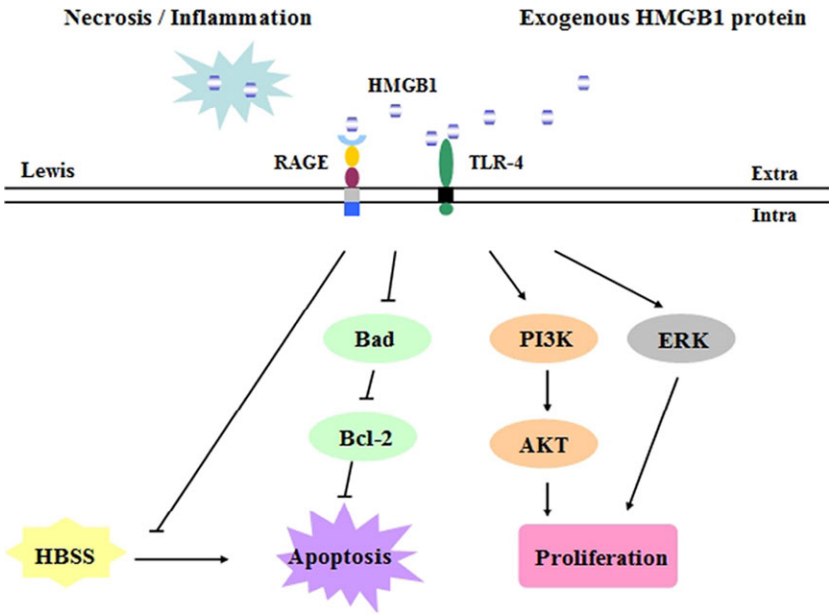

Figure 6 A schematic of HMGB1-RAGE/TLR4 interaction regulated apoptosis and proliferation of Lewis. HMGB1 is released following necrosis/inflammation in cells as DAMPs in the tumour microenvironment, promoting inflammation. HMGB1 binds specific receptor RAGE and TLR4 rescuing apoptosis of Lewis induced by HBSS starvation, inhibiting the expression of pro-apoptosis protein Bad and promoting the expression of anti-apoptosis protein Bcl-2. This means HMGB1 can protect cells from apoptosis. Meanwhile, HMGB1 also stimulates phosphorylation of PI3K/Akt and Erk1/2. As shown in the Conclusion, the HMGB1–RAGE/TLR4 pathway inhibits apoptosis and enhances growth of tumour cells via signal pathways of PI3K/Akt and Erk. Ability to control HMGB1 release from cells or to inhibit its activity appears to be a promising therapeutic approach. Currently, many inhibitors of HMGB1 protein are known and can be used in anticancer therapy. HBSS, Hanks' balanced salt solution; RAGE, receptor for advanced glycation end products.

HBSS starvation by promoting Bcl-2 expression. Furthermore, exogenous HMGB1 protein could induce the Lewis proliferation through HMGB1-RAGE/TLR4-PI3K/Akt pathway or HMGB1-RAGE/TLR4-Erk1/2 pathway (Fig. 6).

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Conflict of interest

None to declare.

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