

HMGB1 release by human liver L02 and HepG2 cells induced by lipopolysaccharide

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Abstract. Liver cells release the high mobility group box-1 (HMGB1) protein when exposed to lipopolysaccharides (LPSs). However, the timing and levels of protein released remain unclear. The present study aimed to characterize the secretion of the late pro-inflammatory cytokine HMGB1 by liver L02 and HepG2 cells. The human mononuclear macrophage cell line U937 was used as a control. Various concentrations of LPS were added to human U937, L02 and HepG2 cells for different durations, and the cells were analyzed at different time-points following this addition. Reverse transcription polymerase chain reaction (RT-PCR) was used to measure cellular HMGB1 mRNA levels, western blotting was performed to detect HMGB1 in cellular supernatants and the translocation of HMGB1 from the nucleus to the cytosol was examined using immunofluorescence staining. L02 and HepG2 cells exhibited higher HMGB1 mRNA levels compared with the control U937 cells 20 and 24 h following continuous exposure to LPS. U937 cells exhibited higher HMGB1 mRNA levels compared with the corresponding L02 and HepG2 cells 16 h following LPS exposure. The phase of HMGB1 protein detected in the cellular supernatants of L02 and HepG2 cells (16 h) was later than that of U937 cells (8 h). For the three cell lines, HMGB1 levels demonstrated a time dependency; however, the protein level was the highest in U937 cells. In the three cell lines, translocation of HMGB1 from the nucleus to the cytosol occurred; however, the phases

of HMGB1 translocation in L02 and HepG2 cells occurred later than in U937 cells. LPS-induced secretion of the late pro-inflammatory cytokine HMGB1 by liver cells is characterized by a late phase of release and smaller quantity, and the process of HMGB1 secretion appears to be associated with HMGB1 translocation.

Introduction

High mobility group box-1 (HMGB1) protein, discovered in the 1970s, is a non-histone nucleoprotein, rich in eukaryotes. It has a highly conserved structure; the homology of the amino acid sequence between humans and rodents is >99% (1). HMGB1 is widely distributed across different organs, including the lymph, heart, liver, kidney, lung and brain tissues of various species, and has evolved to be a substance necessary for life. Studies have demonstrated that *HMGB1* knock-out mice die several hours following their birth (2). The localization of HMGB1 in the nucleus is determined by two lysine-rich nuclear localization sequences (NLS). The N-terminus of HMGB1 contains two repeats of positively charged domains (A box and B box), and a negatively charged C-terminus; these domains allow HMGB1 to bind to chromatin DNA, thus participating in DNA repair, replication, transcription and translation and the regulation of various cellular activities (3-5). The function of HMGB1 is related to its localization. HMGB1 in the nucleus participates in gene expression and regulation (6), whereas dynamically moving HMGB1 in the cytoplasm and nucleus is involved in regulating axon growth, the taxis of smooth muscle cells and the transfer of tumor cells (7,8). Recent studies have demonstrated that when nuclear HMGB1 is released into the extracellular space, it serves as an inflammatory mediator that participates in numerous pathological and physiological processes, maintaining and augmenting inflammatory responses (9). Thus it has a key role in the complex cytokine secretion and regulatory network (10).

It is necessary for HMGB1 to be released into the extracellular space in order to exert an inflammatory effect. In the

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extracellular space, HMGB1 is a key 'late' pro-inflammatory cytokine. In 1999, Wang *et al* reported the important role of HMGB1 in sepsis (9). In recent years, there have been an increasing number of studies demonstrating that HMGB1 is involved in various diseases. As a pro-inflammatory cytokine, the pathogenic action of HMGB1 has two prominent characteristics. Firstly, the time at which HMGB1 takes effect is relatively late. Unlike early pro-inflammatory cytokines, such as the commonly seen tumor necrosis factor- α (TNF- α), HMGB1 is not released into the extracellular space to exert its inflammatory effect until at least 20 h following stimulation (2,9). However, this is not the case in ischemia-reperfusion injuries (11); it has been demonstrated that in ischemia-reperfusion models, HMGB1 release occurs within 4 h of stimulation, although the mechanism of this process remains unclear. Secondly, HMGB1 is capable of sustaining and augmenting the inflammatory response. It serves as an inflammatory cytokine that participates in signal transduction and also interacts with multiple pro-inflammatory cytokines to mutually induce expression (12); it may also lead to the nuclear translocation of the inflammatory signal molecule nuclear factor (NF)- κ B (13,14). HMGB1 contributes to a complex cytokine secretion and regulatory network that initiates, sustains and augments the inflammatory response. HMGB1 is a key component of the inflammatory cytokine network (10), not only due to the cascade effect of its own secretions, but also due to its role in regulating the secretions of other inflammatory factors. HMGB1 has two methods of release; active 'secretion' (15) by activated immune cells and passive 'leaking' from apoptotic cells (16). HMGB1 secretion by activated immune cells has been widely studied, however there have been relatively few studies with regard to HMGB1 secretion by non-apoptotic cell entities. Previous studies by our group have found that under TNF- α and LPS stimulation, a large amount of HMGB1 may be detected in the supernatants of liver cell cultures (17,18), consistent with the results of Tsung *et al* (19). This was a novel finding for the physiological function of the liver. However, the process of HMGB1 release by liver cells remains unclear. In this study, HMGB1 release by the human mononuclear macrophage cell line U937 was used as a reference, and the release of the late pro-inflammatory cytokine HMGB1 by apoptotic liver cells was investigated.

Materials and methods

Cell lines. Human mononuclear macrophage cell line U937 and human liver cell lines L02 and HepG2 were purchased from the cell bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China).

Study design. Cell lines L02, HepG2 and U937 were regularly cultured. The number of cells was adjusted to 2.0×10^6 , and the cells were transferred to opti-MEM1 low-serum medium (Gibco, Carlsbad, CA, USA) for 4 h. Subsequently, LPS at a specific final concentration was added and cellular supernatants were collected at various time-points (0, 4, 8, 12, 16, 20 and 24 h). Western blotting was performed to measure the HMGB1 protein level and a methyl thiazolyl tetrazolium (MTT) assay was performed in order to detect the survival rate of the cells. HMGB1 mRNA levels were measured

by reverse transcription polymerase chain reaction (RT-PCR), apoptosis was detected using a fluorescent TUNEL assay (Roche, USA) and HMGB1 translocation was examined by immunofluorescence. Three independent measurements were performed and results were averaged.

MTT assay. L02, HepG2 and U937 cells were cultured in 96-well plates and treated with varying concentrations of LPS for different durations. MTT (20 μ l; Sigma, St. Louis, MO, USA) was added to each well and cultured in an incubator at 37°C for 4 h. The supernatant was then discarded and 150 μ l DMSO was added to each well and agitated for 10 min. An automated ELISA analyzer (Chondrex, USA) was used for chromatometry. For the corresponding control group, normal saline (NS) of the same volume as LPS was added; DMSO and a low-serum medium were used for the baseline group. The cell survival rate was calculated according to the following formula: survival rate = (OD experimental group - OD baseline group)/(OD control group - OD baseline group).

Measurement of lactic dehydrogenase (LDH) content. Cellular supernatants of the HepG2, L02 and U937 cells were collected at various time-points (0, 4, 8, 12, 16, 20 and 24 h) and centrifuged to remove cell fragments. An Amicon Ultra-4 ml 10 KD ultrafiltration centrifugal tube (Millipore Inc., Billerica, MA, USA) was used to concentrate the solution 20-50 times. LDH contents of the groups were subjected to routine detection at the biochemistry laboratory of Xiangya Hospital, Central South University (Changsha, China).

Detection of apoptosis by fluorescent TUNEL assay. After each of the groups were treated, the cells were fixed with 4% pre-cooled paraformaldehyde, placed on a horizontal shaker at 25°C and subjected to gentle agitation for 60 min. Next, 0.1 ml permeating liquid was added and allowed to settle at 4°C for 10 min; 50 μ l TUNEL reaction mixture (Boehringer Mannheim, Germany) was added to the wells of each experimental group and reacted in the dark for 60 min at 37°C. Freshly prepared DAPI working solution (1-2 drops of 2 mg/l) was added to stain the nuclei and cells were observed under a fluorescent microscope. Negative and positive control wells were also prepared. In the negative control well, the reaction mixture was replaced by Vial 2 solution (Roche) and the well was placed in the dark for 1 h. In the positive control well, following permeation, 3 U/ml DNase I (Sigma) was added and allowed to react for 10 min at 25°C. Subsequently, the reaction mixture was added for the TUNEL reaction.

Detection of HMGB1 mRNA level by RT-PCR. After treating each group, total RNA extraction and RT were performed according to the manufacturer's instructions (Qiagen RNeasy Mini Extraction kit, Qiagen, Inc., Hilden, Germany). The PCR primer was designed using Primer premier 5.0 software based on human HMGB1 and β -actin cDNA sequences in the Genbank. The primers were synthesized by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China) and their sequences were as follows: HMGB1, GAG CAT AAG AAG AAG CAC CCA GAT GGG CGA TAC TCA GAG CAG AAG (target fragment, 257 bp); and β -actin, GAC AGG ATG CAG AAG GAG ATT

ACT TGA TCC ACA TCT GCT GGA AGG T (target fragment, 142 bp). The PCR reaction conditions for HMGB1 were as follows: 94°C for 4 min, 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec, and 32 cycles of 72°C for 7 min. The PCR reaction conditions for β -actin were as follows: 94°C for 5 min, 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec and 30 cycles of 72°C for 7 min. Subsequently, PCR products (5 μ l) were subjected to 2% agarose gel electrophoresis and images were captured under UV light. A bandscan imaging analysis system (Eagle Eye II, Stratagene, USA) was used to scan the bands and the photodensity of each band was acquired. The relative band density of HMGB1 was calculated.

Measurement of HMGB1 protein by western blotting. The cell culture supernatants from each group were collected and the cell fragments were filtered out. An Amicon Ultra-4ml 10 KD ultrafiltration centrifugal tube (Millipore Inc.) was used to concentrate the solution and the Coomassie brilliant blue method (Sigma) was used to measure the protein content. To a 40 μ l sample, a loading buffer of the same volume was added and mixed, and the mixture was subjected to denaturation by boiling. Separating gel (10% SDS-PAGE) and 5% spacer gel were prepared, and the samples were loaded for electrophoresis in an electrophoresis buffer. The gels were electrotransferred to polyvinylidene difluoride (PVDF) membranes. After blocking with a buffer containing 5% skim milk powder for 30 min, rabbit anti-human polyclonal antibody (Abcam, USA; 1:1,000 dilution) was added for an overnight reaction at 4°C. Following washing with Tris-buffered saline with Tween-20 (TBST), the PVDF membranes were incubated with a secondary antibody (Merck, Germany; 1:500 dilution) for 1 h at 37°C. Following a further TBST wash, exposure, development, fixation and positive image production were performed using the ECL Western Blotting Detection kit (Amersham Pharmacia Biotech, Amersham, UK), according to the manufacturer's instructions. The film was scanned and photodensity was analyzed using Quantity One software (Bio-Rad, Hercules, CA, USA). The relative protein content of different samples was calculated based on the known concentration of recombinant human HMGB1.

HMGB1 translocation detection. Following each treatment period, the cell supernatant from each group was discarded. After three washes with PBS, cells were fixed with 4% paraformaldehyde for 15 min. The cells were dried at room temperature, subsequently treated with 0.1% Triton x-100 at 4°C for 10 min and then blocking at room temperature was performed for 30 min using a blocking buffer. The cells were spun dry and rabbit anti-human polyclonal antibody (1:1000 dilution) was added for an overnight incubation at 4°C. Subsequently, following three washes with PBS, a fluorescein isothiocyanate (FITC)-labeled fluorescent secondary antibody (1:750 dilution) was added for incubation at 37°C for 120 min. Following a further three washes with PBS, DAPI (final concentration, 5 μ mol/l) was used for double staining at room temperature for 10 min, and glycerol jelly was used to mount the slides. The slides were examined under a fluorescent microscope, and Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA) was used for semi-quantitative analysis of the relative fluorescence intensity in the nuclei and cytoplasm.

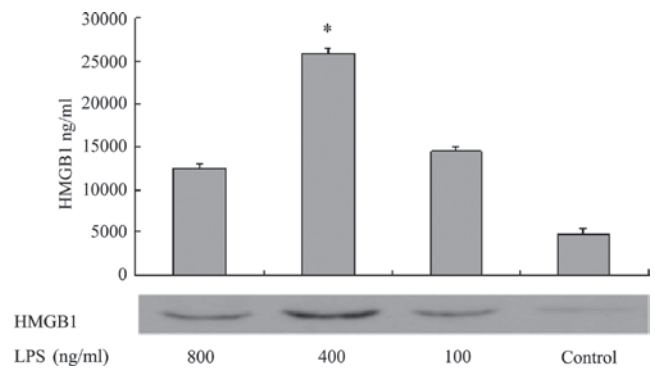


Figure 1. Relative high mobility group box-1 (HMGB1) protein content in the supernatant of L02 cells using western blotting 20 h after the addition of various concentrations of lipopolysaccharide (LPS). *HMGB1 content at 400 ng/ml LPS was significantly higher than that at the other three LPS concentrations ($P < 0.05$, $n = 3$).

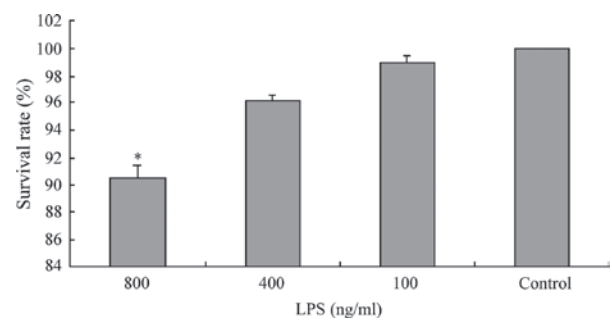


Figure 2. Survival rate (% mean \pm SD) of L02 cells 20 h after the addition of various concentrations of lipopolysaccharide (LPS). *When the concentration of LPS reached 800 ng/ml, the survival rate decreased significantly ($P < 0.05$).

Statistical analysis. The SPSS 13.0 software package was used to analyze and process the data. A t-test was performed for comparison between the groups and analysis of variance (ANOVA) was performed for comparison within a group. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of stimulation with various concentrations of LPS. Western blotting was performed to measure HMGB1 levels in the supernatants of L02 cells 20 h after the addition of various concentrations of LPS. The results demonstrated that when the LPS concentration was in the range of 0-400 ng/ml, the concentration of HMGB1 in the cell culture supernatant increased as the LPS concentration increased. However, when the LPS concentration was > 400 ng/ml, the concentration of HMGB1 in the supernatant no longer increased. At the final LPS concentration of 800 ng/ml, the HMGB1 content was significantly lower than that when the LPS concentration was 400 ng/ml ($P < 0.05$, Fig. 1). The MTT assay measured the survival rates of L02 cells following 20 h of continuous exposure to LPS at various concentrations (100, 400, 800 ng/ml). The results demonstrated that when the LPS concentration was in the range 0-400 ng/ml, the survival rate of L02 cells was $> 95\%$ (99.99 ± 0.01 , 98.97 ± 0.52 and $96.18 \pm 0.34\%$ for the control, LPS 100 ng/ml and 400 ng/ml groups, respectively).

Table I. Survival rates of HepG2, L02 and U937 cells at various time-points following the addition of LPS, measured by an MTT assay (mean \pm SD, n=3).

Group	Survival rate (%)						
	4 h	8 h	12 h	16 h	20 h	24 h	48 h
HepG2	99.30 \pm 0.18	98.63 \pm 0.22	96.98 \pm 0.79	95.47 \pm 2.14	94.24 \pm 0.99	91.01 \pm 1.17	83.54 \pm 1.26 ^a
L02	99.52 \pm 0.29	98.37 \pm 0.89	95.94 \pm 4.13	95.46 \pm 1.73	92.87 \pm 1.79	90.76 \pm 0.91	82.77 \pm 1.06 ^a
U937	99.41 \pm 0.35	97.73 \pm 0.86	96.65 \pm 0.31	94.42 \pm 1.86	91.75 \pm 3.11	91.21 \pm 2.033	81.88 \pm 0.96 ^a

^aAt 48 h following the addition of LPS, the survival rates of cells from all groups significantly decreased ($P < 0.05$). LPS, lipopolysaccharide; MTT, methyl thiazolyl tetrazolium.

Table II. LDH content in the supernatant of cells from different groups at various time-points following the addition of LPS (mean \pm SD, n=3).

Group	LDH content (U/l)						
	4 h	8 h	12 h	16 h	20 h	24 h	
HepG2 + NS	0.0257 \pm 0.0021	0.1428 \pm 0.0024	0.1868 \pm 0.0068	0.2607 \pm 0.0175	0.4234 \pm 0.0563	1.1049 \pm 0.0831	
HepG2 + LPS	0.0274 \pm 0.0018	0.1382 \pm 0.0046	0.1948 \pm 0.0057	0.2768 \pm 0.0368	0.4580 \pm 0.0242	1.1830 \pm 0.0732	
L02 + NS	0.0247 \pm 0.0007	0.1091 \pm 0.0094	0.1162 \pm 0.0054	0.1191 \pm 0.0049	0.4173 \pm 0.0200	1.3330 \pm 0.2947	
L02 + LPS	0.0252 \pm 0.0049	0.1181 \pm 0.0118	0.1212 \pm 0.0022	0.1374 \pm 0.0050	0.4221 \pm 0.0342	1.2616 \pm 0.1579	
U937 + NS	0.0265 \pm 0.0022	0.1369 \pm 0.0021	0.1350 \pm 0.0092	0.1354 \pm 0.0142	0.4365 \pm 0.0096	1.7306 \pm 0.0373	
U937 + LPS	0.0294 \pm 0.0024	0.1398 \pm 0.0086	0.1454 \pm 0.0232	0.1477 \pm 0.0199	0.4487 \pm 0.0288	1.5532 \pm 0.2679	

There was no significant difference between any of the LPS-treated and corresponding control groups ($P > 0.05$). LDH, lactic dehydrogenase; LPS, lipopolysaccharide; NS, normal saline.

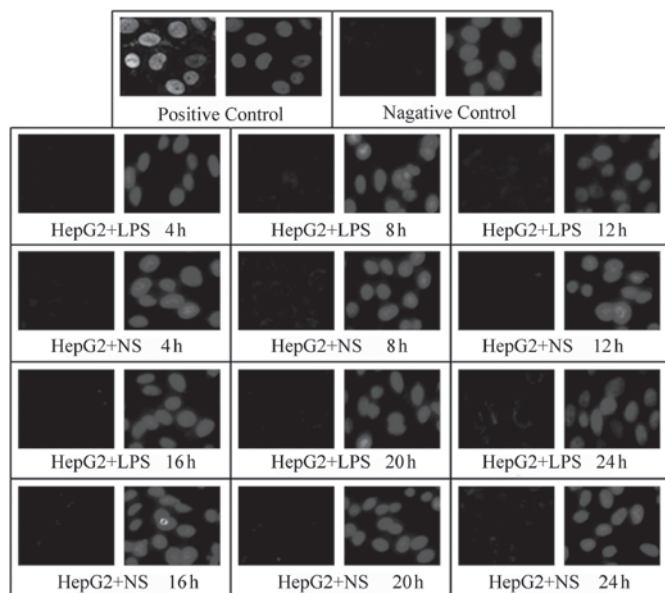


Figure 3. Effect of lipopolysaccharide (LPS) stimuli on HepG2 apoptosis, as determined by a TUNEL assay. At 0-20 h, neither cells from LPS-treated groups nor those from the corresponding control groups demonstrated notable fluorescence; at 24 h, a small amount of non-specific fluorescence was observed. Semi-quantitative analysis demonstrated that there was no significant difference between the rates of apoptosis in the experimental and control groups ($P > 0.05$).

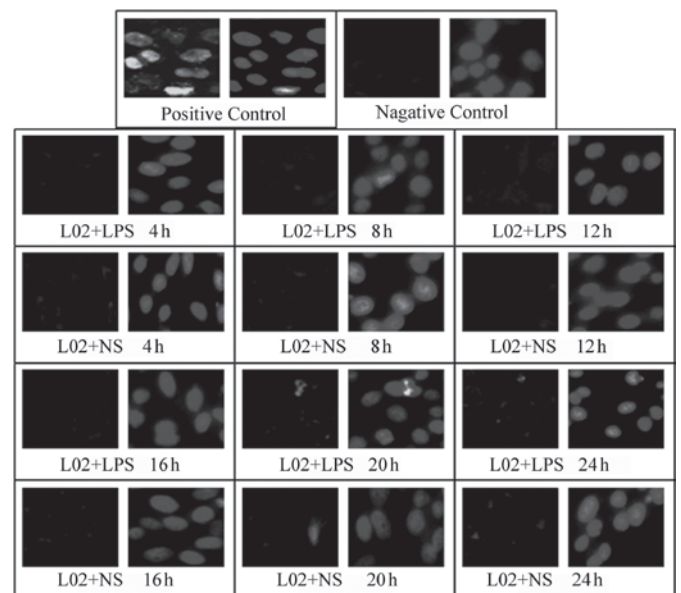


Figure 4. Effect of lipopolysaccharide (LPS) stimuli on L02 apoptosis, as determined by a TUNEL assay. At 0-16 h, neither cells from LPS-treated groups nor those from the corresponding control groups demonstrated notable fluorescence; at 20 and 24 h a small amount of non-specific fluorescence was observed. Semi-quantitative analysis demonstrated that there was no significant difference between the rates of apoptosis rate in the experimental and control groups ($P > 0.05$).

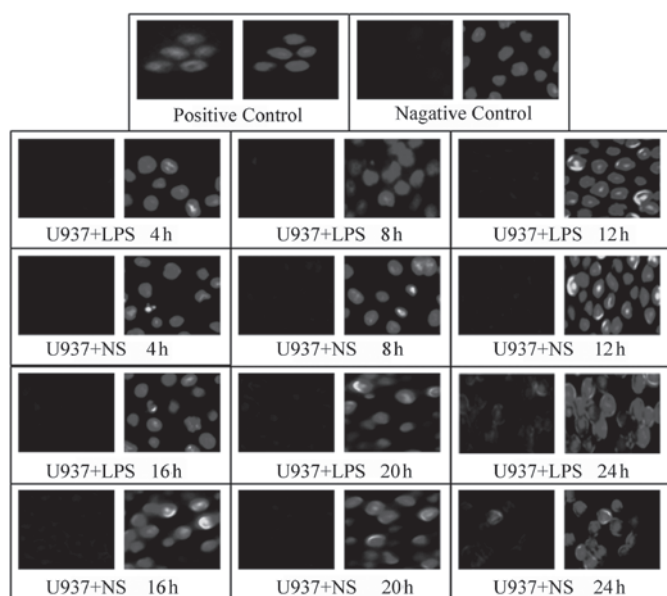


Figure 5. Effect of lipopolysaccharide (LPS) stimuli on U937 apoptosis, as determined by a TUNEL assay. At 0-20 h, neither cells from LPS-treated groups nor those from the corresponding control groups exhibited notable fluorescence; at 24 h a small amount of non-specific fluorescence was observed. Semi-quantitative analysis demonstrated that there was no significant difference between the apoptosis rates in any of the experimental and control groups ($P>0.05$).

However, when the concentration of LPS was increased to 800 ng/ml, the survival rate of the L02 cells significantly decreased to $90.59\pm 0.76\%$ (Fig. 2).

Survival rates of HepG2, L02 and U937 cells at various time-points following the addition of LPS. As shown in Table I, at 0-24 h following the addition of LPS, the survival rates of HepG2, L02 and U937 cells were all $>90\%$. However, at 48 h, the survival rates significantly decreased ($P<0.05$), and the level of HMGB1 released passively by apoptotic cells also increased. Therefore, we selected 0-24 h following treatment with LPS to be the range for the measurement of damage-related factor LDH, apoptosis detection, measurement of HMGB1 mRNA levels using RT-PCR and the measurement of HMGB1 protein levels using western blotting.

LDH contents in the supernatants of HepG2, L02 and U937 cells at various time-points following the addition of LPS. The LDH content in the supernatants of HepG2, L02 and U937 cells were measured at different time-points (0-24 h) following the addition of 400 ng/ml LPS. The results demonstrated that the LDH content in the cell supernatants of different groups gradually increased with time; for each group, the LDH content in the supernatant at 24 h was significantly higher than that at 0-20 h ($P<0.05$). However, at each time-point, there was no significant difference in the LDH content in the cell supernatants between the LPS-treated and corresponding control groups ($P>0.05$; Table II).

Effect of LPS stimulation on the apoptosis of HepG2, L02 and U937 cells. A TUNEL assay was used to detect the effect of LPS (400 ng/ml) stimulation on the apoptosis of HepG2, L02

Table III. Relative fluorescence intensity indicating apoptosis in cells from different groups at various time-points following the addition of LPS (mean \pm SD, $n=3$), measured by fluorescence TUNEL assay.

Group	Fluorescence intensity (U/I)									
	Positive control	Negative control	0 h	4 h	8 h	12 h	16 h	20 h	24 h	
L02 + LPS	6.3640 \pm 0.6084	0.0660 \pm 0.0261	0.0753 \pm 0.0246	0.0647 \pm 0.0260	0.0615 \pm 0.0112	0.0790 \pm 0.0197	0.0937 \pm 0.0091	0.0960 \pm 0.0313	0.1393 \pm 0.0325	
L02 + NS	6.3640 \pm 0.6084	0.0660 \pm 0.0261	0.0607 \pm 0.0117	0.0780 \pm 0.0197	0.0740 \pm 0.0236	0.0743 \pm 0.0431	0.0877 \pm 0.0423	0.0947 \pm 0.0320	0.1270 \pm 0.0201	
HepG2 + LPS	7.4211 \pm 0.7480	0.0377 \pm 0.0172	0.1047 \pm 0.0187	0.1257 \pm 0.0493	0.1190 \pm 0.0468	0.1213 \pm 0.0199	0.1320 \pm 0.0295	0.1047 \pm 0.0240	0.2143 \pm 0.0991	
HepG2 + NS	7.4211 \pm 0.7480	0.0377 \pm 0.0172	0.0920 \pm 0.0166	0.1287 \pm 0.0252	0.1370 \pm 0.0275	0.1380 \pm 0.0556	0.1300 \pm 0.0393	0.1307 \pm 0.0335	0.2347 \pm 0.0735	
U937 + LPS	4.9257 \pm 1.1101	0.0400 \pm 0.0190	0.1303 \pm 0.0345	0.1433 \pm 0.0545	0.1470 \pm 0.0578	0.1433 \pm 0.0539	0.1673 \pm 0.0524	0.1820 \pm 0.0174	0.2133 \pm 0.0467	
U937 + NS	4.9257 \pm 1.1101	0.0400 \pm 0.0190	0.1127 \pm 0.0327	0.1400 \pm 0.0423	0.1443 \pm 0.0485	0.1420 \pm 0.0651	0.1707 \pm 0.0748	0.1920 \pm 0.0217	0.2077 \pm 0.0142	

There was no significant difference between various time-points for the same type of cell; there was also no significant difference between the LPS-treated and the corresponding control groups at the same time-points ($P>0.05$). LPS, lipopolysaccharide. NS, normal saline.

Table IV. Relative fluorescence intensity of cells from different groups at various time-points following the addition of LPS (mean \pm SD, n=3).

Group	Fluorescence intensity (%)						
	0 h	4 h	8 h	12 h	16 h	20 h	24 h
L02 + LPS							
N	64.58 \pm 3.11..	63.95 \pm 4.66..	64.16 \pm 1.52..	49.75 \pm 3.03	39.24 \pm 3.20	28.48 \pm 5.15	22.36 \pm 3.38
C	5.16 \pm 1.43	4.65 \pm 0.59	3.90 \pm 1.28	19.04 \pm 1.42 ^a	28.83 \pm 3.61 ^a	42.04 \pm 9.54 ^a	34.07 \pm 5.12 ^a
L02 + NS							
N	63.09 \pm 3.83..	62.21 \pm 5.44..	64.05 \pm 4.03..	60.93 \pm 8.93	61.37 \pm 7.68	56.85 \pm 6.98	49.85 \pm 6.47
C	4.52 \pm 0.56	4.37 \pm 1.54	4.32 \pm 0.99	5.68 \pm 1.17	7.05 \pm 1.47	9.74 \pm 2.87	14.40 \pm 4.07
HepG2 + LPS							
N	69.13 \pm 1.69..	68.38 \pm 3.21..	68.20 \pm 2.93..	59.48 \pm 3.81	44.32 \pm 4.46	34.21 \pm 3.40	26.70 \pm 7.32
C	3.65 \pm 0.67	3.86 \pm 1.39	4.55 \pm 1.81	14.74 \pm 1.34 ^a	27.06 \pm 3.59 ^a	45.51 \pm 5.16 ^a	33.67 \pm 3.86 ^a
HepG2 + NS							
N	69.87 \pm 4.72..	71.17 \pm 4.28..	71.05 \pm 1.81..	70.77 \pm 8.18	67.52 \pm 7.74	63.77 \pm 5.07	51.95 \pm 5.93
C	3.14 \pm 1.50	3.62 \pm 1.67	4.77 \pm 2.42	4.73 \pm 1.66	5.89 \pm 2.54	7.72 \pm 2.70	16.29 \pm 3.01
U937 + LPS							
N	52.47 \pm 3.61..	50.30 \pm 4.36..	42.50 \pm 1.80..	35.41 \pm 2.47	30.07 \pm 2.55	25.65 \pm 3.51	23.96 \pm 4.64
C	1.90 \pm 0.65	3.94 \pm 1.15	10.91 \pm 1.17 ^b	19.35 \pm 1.50 ^b	25.44 \pm 1.06 ^b	34.31 \pm 2.97 ^b	26.67 \pm 3.87 ^b
U937 + NS							
N	53.45 \pm 4.31..	52.77 \pm 6.76..	52.45 \pm 5.17..	53.51 \pm 3.10	51.40 \pm 6.34	48.38 \pm 7.22	42.26 \pm 7.17
C	1.95 \pm 1.69	1.87 \pm 0.99	2.30 \pm 1.21	2.16 \pm 0.57	2.8 \pm 0.67	6.98 \pm 1.51	12.77 \pm 1.24

Semi-quantitative fluorescence analysis showed that at 12-24 h, the fluorescence intensities in the cytoplasm of HepG2 and L02 cells from the LPS-treated group were significantly higher than those in the corresponding controls (^aP<0.05); whereas in U937 cells, starting at 8 h, the fluorescence intensity in the cytoplasm of cells from the LPS-treated group was significantly higher than in the corresponding controls (^bP<0.05). LPS, lipopolysaccharide; NS, normal saline; N, nuclei; C, cytoplasm.

and U937 cells at various time-points following the addition of LPS (4, 8, 12, 16, 20 and 24 h); NS of the same volume was added to the corresponding control groups. In the positive control, green fluorescence was observed in complete or broken nuclei and blue fluorescence in the same field of view marked cell nuclei double-stained by DAPI. As shown in Figs. 3-5, it was observed that there was little non-specific fluorescence in the HepG2, L02 and U937 cells at various time-points (high magnification). The relative fluorescence intensities of various cells are shown in Table III; there were no significant differences between the different time-points. Furthermore, at the same time-points, there were no significant differences between the LPS-treated and corresponding control groups (P>0.05).

Effect of LPS stimulation on HMGB1 translocation in HepG2, L02 and U937 cells. An immunofluorescence assay was used to examine HMGB1 translocation in HepG2, L02 and U937 cells at different time-points following the addition of LPS (400 ng/ml; 0, 4, 8, 12, 16, 20 and 24 h). Under normal conditions, HMGB1 in HepG2, L02 and U937 cells was concentrated in the nuclei. Following treatment with 400 ng/ml LPS, green fluorescence from specifically labeled HMGB1 was observed at 12 h in the cytosol of HepG2 and L02 cells; the fluorescence in the cytoplasm increased, whereas that in the nuclei decreased

with time, suggestive of the translocation of HMGB1 from the nuclei to the cytoplasm. In the corresponding control group, a small amount of fluorescence was observed in the cytoplasm, but the majority remained in the nuclei (Figs. 6 and 7). Relative fluorescence intensities of the groups were analyzed further by Image-Pro Plus software and are shown in Table IV. The results revealed that compared with 16 and 20 h following the addition of LPS, the total fluorescence at 24 h in the cytoplasm and nuclei of HepG2 and L02 cells was significantly reduced (P<0.05). Furthermore, at 0, 4 and 8 h following the addition of LPS, there was no difference in fluorescence intensity in the cytoplasm or nuclei of cells between the LPS-treated HepG2 or L02 group and the corresponding control group (P>0.05). However, at 12-24 h, the fluorescence intensities in the cytoplasm of cells from the LPS-treated HepG2 and L02 groups were significantly higher than those of the corresponding control groups (P<0.05).

The translocation of HMGB1 from the nuclei to the cytoplasm in U937 cells occurred earlier than in HepG2 and L02 cells; at ~4 h, green fluorescence was observed in the cytoplasm and at 8 h, a substantial degree of green fluorescence was observed in the cytoplasm of U937 cells. At 20 and 24 h, the green fluorescence in the cytoplasm was markedly decreased. For cells of the control group (NS-treated), the green fluores-

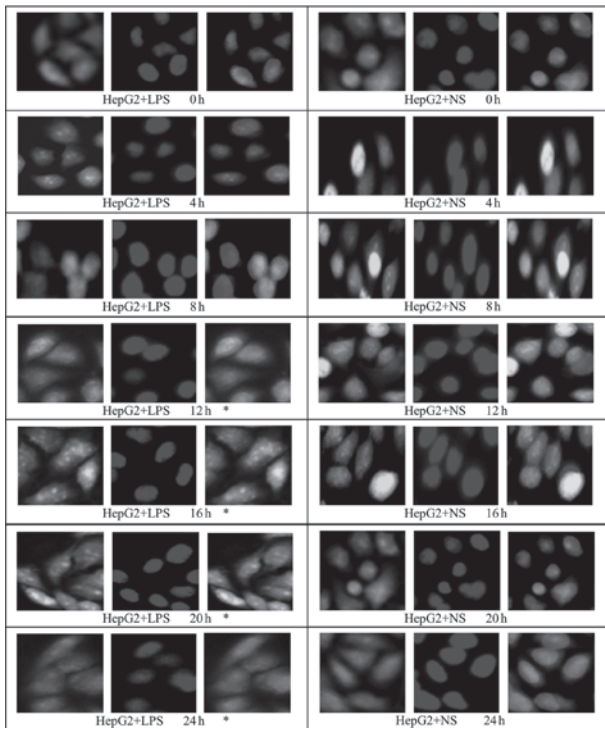


Figure 6. High mobility group box-1 (HMGB1) translocation in HepG2 cells, as detected by an immunofluorescence assay at various time-points following the addition of lipopolysaccharide (LPS; 400 ng/ml; magnification, x200). At 12-24 h, substantial fluorescence was detected in the cytosol of LPS-treated HepG2 cells; however, in the corresponding control group, fluorescence was mainly detected in the nuclei. Semi-quantitative analysis revealed that this difference was significant ($P<0.05$).

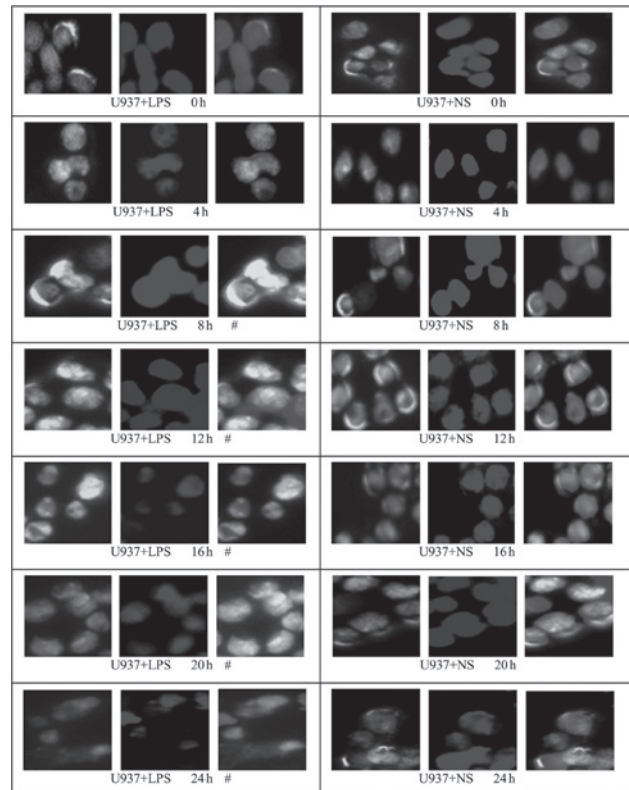


Figure 8. High mobility group box-1 (HMGB1) translocation in U937 cells, as detected by an immunofluorescence assay at different time-points following the addition of lipopolysaccharide (LPS; 400 ng/ml; magnification, x400). At 4 h, green fluorescence was observed in the cytosol of LPS-treated U937 cells and at 8-24 h, substantial fluorescence was detected in the cytosol of LPS-treated U937 cells; however, in the corresponding control group, fluorescence was mainly detected in the nuclei. Semi-quantitative analysis revealed that these differences were significant ($^{\#}P<0.05$).

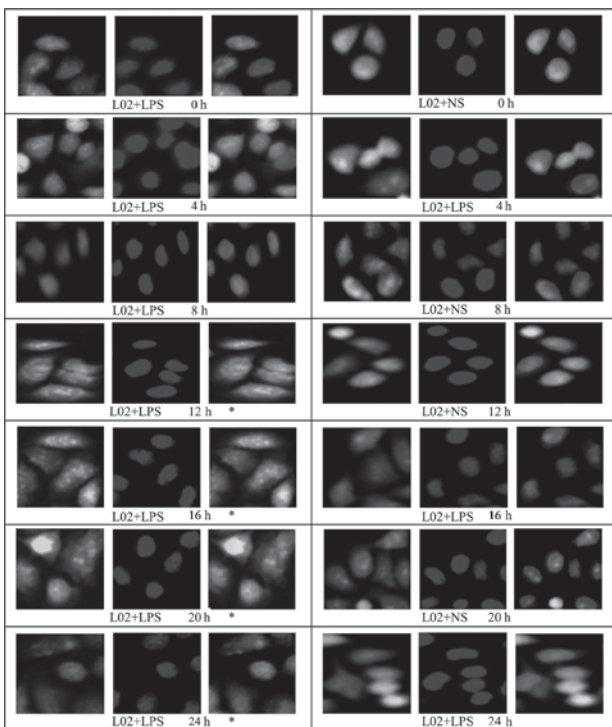


Figure 7. High mobility group box-1 (HMGB1) translocation in L02 cells, as detected by an immunofluorescence assay at various time-points following the addition of lipopolysaccharide (LPS; 400 ng/ml; magnification, x200). At 12-24 h, substantial fluorescence was detected in the cytosol of LPS-treated L02 cells, whereas in the corresponding control group, fluorescence was mainly detected in the nuclei. Semi-quantitative analysis revealed that this difference was significant ($P<0.05$).

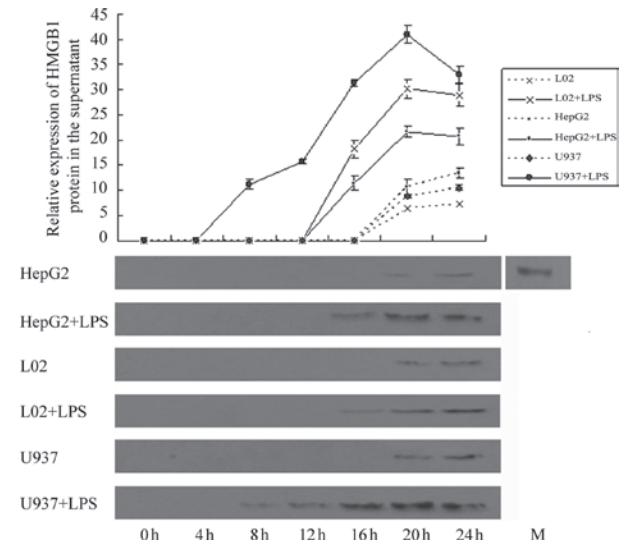


Figure 9. Effect of lipopolysaccharide (LPS) stimuli on high mobility group box-1 (HMGB1) secretion by HepG2, L02 and U937 cells. At 8-24 h, HMGB1 content in the supernatant of U937 cells was significantly higher than that in the corresponding control ($P<0.05$). At 16-24 h, HMGB1 content in the supernatants of HepG2 and L02 cells was significantly higher than that in the corresponding controls ($P<0.05$). At 20 and 24 h, an extremely small amount of HMGB1 was detected in the three control groups (treated with normal saline; NS) and these amounts were significantly lower than those of the corresponding experimental groups ($P<0.05$). Comparison of the HMGB1 secretion levels from the three cell types at the same time-points following LPS treatment demonstrated that U937 cells secreted the highest level of HMGB1. M, reference.

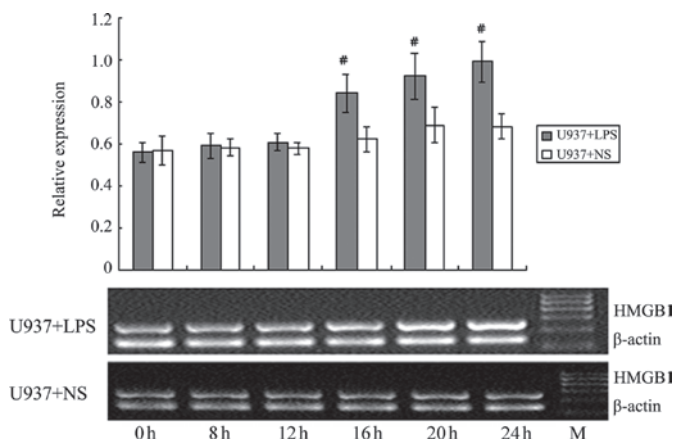


Figure 10. Effect of lipopolysaccharide (LPS) stimuli on high mobility group box-1 (HMGB1) mRNA levels in U937 cells. *Comparison with corresponding control group (NS-treated; $P < 0.05$). NS, normal saline; M, reference.

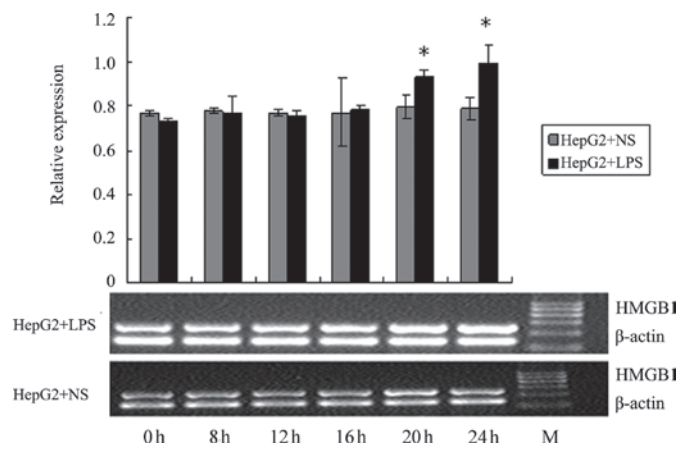


Figure 12. Effect of lipopolysaccharide (LPS) stimuli on high mobility group box-1 (HMGB1) mRNA levels in HepG2 cells. *Comparison with corresponding control group (NS-treated; $P < 0.05$). NS, normal saline; M, reference.

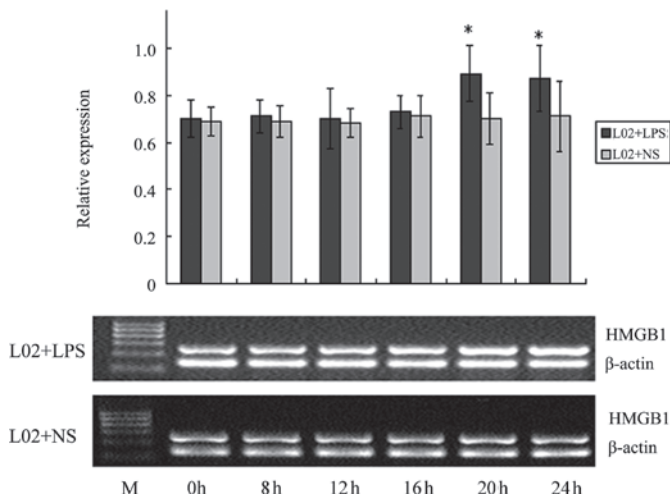


Figure 11. Effect of lipopolysaccharide (LPS) stimuli on high mobility group box-1 (HMGB1) mRNA levels in L02 cells. *Comparison with corresponding control group (NS-treated; $P < 0.05$). NS, normal saline; M, reference.

cence, representing specifically labeled HMGB1, was localized within the nucleus and no marked translocation was observed. Since the cytoplasm of U937 cells is relatively small, the cytoplasmic staining of U937 cells was less conspicuous than that of the HepG2 and L02 cells (Fig. 8). Relative fluorescence intensities of the different groups were further analyzed by Image-Pro Plus software and are shown in Table IV; from 8 h, the fluorescence intensity in the cytoplasm of cells from the LPS-treated U937 group was significantly higher than that of the corresponding control group ($P < 0.05$).

Effect of LPS stimulation on HMGB1 release by HepG2, L02 and U937 cells. Western blotting results (Fig. 9) revealed that at 4 h following the addition of LPS (400 ng/ml), no HMGB1 band was detected in the supernatants of HepG2, L02 and U937 cells. At 8 h, a weak HMGB1 band was observed in the supernatant of U937 cells and at 16 h, a weak HMGB1 band was detected in the supernatants of HepG2 and L02 cells. The photodensities of the HMGB1 bands from the three types of cells gradually increased with time. Semi-quantitative anal-

ysis revealed that at 20 h, HMGB1 content in the cell culture supernatants of the three cell types peaked. At 20 and 24 h, a small quantity of HMGB1 was detected in the cell culture supernatant from the control groups (NS-treated), and this quantity differed significantly from the amount of HMGB1 in the cell supernatant of the corresponding experimental group for the three types of cells ($P < 0.05$). Comparison of the quantity of HMGB1 secreted by the three cell types following LPS treatment demonstrated that HMGB1 secretion by U937 cells was by far the highest, and the differences were significant ($P < 0.05$). Further comparisons revealed that the amount of HMGB1 secreted by L02 cells at 16 h was comparable to that by U937 cells at 13 h, and the amount of HMGB1 secreted by L02 cells at 20 h was comparable to that by U937 cells at 16 h. With increasing time, the amount of HMGB1 secreted by L02 cells at 24 h was significantly lower than that by U937 cells at 20 h ($P < 0.05$). The amount of HMGB1 secreted by HepG2 cells at 20 h was comparable to that by U937 cells at 14 h. With an increase in time, the amount of HMGB1 secreted by HepG2 cells at 24 h was significantly lower than that by U937 cells at 20 h ($P < 0.05$).

Effect of LPS stimulation on HMGB1 mRNA levels in HepG2, L02 and U937 cells. RT-PCR was used to detect the HMGB1 mRNA levels in HepG2, L02 and U937 cells at different time-points following the addition of 400 ng/ml LPS (0, 4, 8, 12, 20 and 24 h). NS of the same volume was added to the corresponding control groups. As shown in Figs. 10-12, at 20 and 24 h following the addition of LPS, HMGB1 mRNA levels in HepG2 and L02 cells were significantly higher than those in the corresponding controls. For U937 cells, the HMGB1 mRNA level at 16 h was already significantly higher than the corresponding control. The increase in HMGB1 mRNA level induced by LPS in U937 cells occurred earlier than in HepG2 and L02 cells, and the magnitude of the increase was also higher; these differences were all deemed to be significant ($P < 0.05$).

Discussion

Under normal conditions, HMGB1 is localized in the nucleus. Thus, the release of HMGB1 from the intracellular to extra-

cellular space is a prerequisite for its extracellular functions. There are two ways by which HMGB1 may be released; active 'secretion' by activated immune cells and passive 'leaking' from apoptotic cells. Previous studies have demonstrated that non-apoptotic HepG2 cells are capable of releasing HMGB1 (17,18). In this study, we characterized the secretion of late pro-inflammatory cytokine HMGB1 by liver L02 and HepG2 cells.

LPS-induced HMGB1 release in liver cells was not caused by cell damage. LDH is a damage-related factor; when cultured cells are damaged, a large quantity of LDH may be detected in the culture fluid. Our results revealed that at all time-points, the LDH content in the cell supernatants of every LPS-treated group was not significantly different from that of the corresponding controls, suggesting that at 0-24 h following the addition of 400 ng/ml LPS, none of the cells had incurred substantial damage.

Liver cells (L02, HepG2) and U937 cells release HMGB1 when treated with LPS; however, their release characteristics vary. The first difference is reflected in the timing of the release. For U937 cells, at 8 h following the introduction of LPS, the HMGB1 band was detected from the cell culture supernatant, whereas for liver cells (L02, HepG2), a weak HMGB1 band was detected from the supernatant at only 16 h following stimulation, suggesting that HMGB1 release from the liver cells occurs substantially later than from the immune cell line U937. The second difference is that the amount of HMGB1 released from the control and liver cells differed. Although the amount of HMGB1 detected in the supernatant of LPS-treated U937 cells was relatively small, it remained larger compared with that in the liver cells (L02, HepG2). RT-PCR results revealed that at 20 and 24 h following the addition of LPS, HMGB1 mRNA levels in the liver cells (L02, HepG2) were significantly higher than those in the corresponding controls. In U937 cells, starting from 16 h, the HMGB1 mRNA levels were already significantly higher than those in the corresponding controls, suggesting that the elevation in HMGB1 mRNA levels occurred earlier in U937 cells vs. liver cells (L02, HepG2). When considering the combined RT-PCR and western blotting results, we noted that for U937 and liver cells (L02, HepG2) at the same effective LPS concentration, the time at which the HMGB1 mRNA level became elevated was later than the time at which the HMGB1 level in the supernatant became elevated, suggesting that increased levels of HMGB1 in the supernatant were not solely from transcription-translation of the increased HMGB1 mRNAs in response to LPS stimulation. The immunofluorescence assay results demonstrated that HMGB1 translocation from the nucleus to the cytoplasm was observed at 12 h in liver cells (L02, HepG2); however, in U937 cells, such translocation occurred at 4-8 h, which was substantially earlier than in the liver cells (L02, HepG2). We speculate that at 0-24 h, the main source of HMGB1 in the supernatant was released from the HMGB1 pool in the nuclei, and was not the result of the regulation of the internal signaling pathway (transcription-translation) in response to stimulation. However, as time progressed, the latter may gradually play a dominant role.

Our study demonstrates that living, non-apoptotic liver cells are capable of secreting and releasing the late inflammatory cytokine HMGB1, and the mechanism of this secretion may be closely associated with the translocation of HMGB1 in the

nuclei. Compared with HMGB1 secretion from immune cells, HMGB1 secretion from liver cells occurs later and in a smaller quantity. These results provide an experimental basis for a more complete understanding of the physiological function of the liver. During the development of severe hepatitis, immune cells of the liver, including mononuclear macrophage cells and NK cells, are activated and release inflammatory cytokines, leading to a second attack on the liver that aggravates the damage (20). The results from this study reveal that non-apoptotic liver cells are capable of releasing HMGB1, which further exacerbates the aforementioned inflammatory response cascade. However, HMGB1 release to the extracellular space may also serve as a warning signal to nearby liver cells and affect the taxis of endothelial cells in order to promote the aggregation of endothelial cells and tissue repair. Hence, HMGB1 release by liver cells in response to external stimuli may also be an important step in the self-protection mechanism of the body. This study provides a new approach to investigating the pathogenic mechanism of severe hepatitis. Furthermore, due to the characteristic functions of HMGB1 in the inflammatory network, it may be a new target for the clinical treatment of severe hepatitis.

In conclusion, LPS-stimulated liver cells are capable of secreting the late inflammatory cytokine HMGB1. Compared with HMGB1 release by immune cells, HMGB1 release by liver cells occurs later and in a smaller quantity. Furthermore, this release is associated with HMGB1 translocation and is not dependent on cell death or apoptosis.

Acknowledgements

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