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Original article

The role of heat shock factor 1 in transcriptional regulation of high mobility group box

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Background: Previous study suggests that high mobility group box 1 (HMGB1) can be a potential late inflammatory mediator. However, whether heat shock factor 1 (HSF1) regulate HMGB1 expression via binding to heat shock element (HSE) is not known.

Objective: We investigated the role of HSF1 in the transcriptional regulation of HMGB1 protein.

Methods: A probe that included HMGB1 promoter region containing HSE was synthesized for electrophoretic mobility shift assay (EMSA) to determine the binding of HSF1 and HSE in the promoter region of HMGB1 gene. Mutant mouse HMGB1 promoter was prepared by PCR amplification on a template of wild-type plasmid DNA with site-directed mutant primers. The mutant DNA fragments were also inserted into a corresponding plasmid. In addition, luciferase reporter plasmids of HMGB1 promoter were constructed to transfect RAW264.7 cells. After that, luciferase activity was measured to assay the effects of the HSF1 transfection on the promoter activity.

Results: EMSA result showed a retardation strap after the coculture of biotin labeled HSF1 binding fragment and nuclear protein extracts. The retardation phenomenon could be competed by unlabeled probe and not by unlabeled mutant probe. A super retardation strap was present after adding HSF1 monoclonal antibody. After the HSE core sites was mutated, the relative luciferase activity of the mutant plasmid decreased by 4.26 folds compared with that in the wild-type (23.54±1.68 vs.100.25±3.26, p <0.01). EMSA assay also confirmed that there were HSF1 binding sites HSE (-668bp~-651bp) in the promoter region of HMGB1. The mutation of the core base of HSF1 binding sites decreased the transcriptional activity of HMGB1.

Conclusion: HSF1 can bind to the promoter region HSE (-668bp~-651bp) of HMGB1, which down-regulates the expression of HMGB1.

Keywords: Heat shock transcription factor 1, high mobility group box 1, mouse, RAW264.7, transcription

In 1999, Wang et al. first reported that high mobility group box 1 (HMGB1) participated in the whole sepsis process as a new, potential late inflammatory mediator [1]. It was also found that high mobility group protein 1 (HMG1) could be secreted into the extracellular environment when the cells were stimulated by LPS. This mediated the lethal effects of lipopolysaccharide (LPS) [2-4]. In view of dynamic characteristics of late expression of HMGB1 and antagonize HMGB1, it may have the potential to treat sepsis.

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In our previous study, we found that severe burn activated various protective mechanisms for heat shock response (HSR), thereby regulating the expressions and releases of inflammatory mediators such as TNF-α, HMGB1, and IL-10 [5]. Heat shock factor 1 (HSF1) can specifically bind to heat shock element (HSE) of IL-10 promoter (-376bp~-369bp). Relative luciferase activity analysis showed that HSF1 could transcribe and activate the IL-10 expression [5]. IL-10 is a major anti-inflammatory factor [6-8]. It suggests that heat shock pre-conditioning may protect against sepsis-induced injury via regulating the IL-10 expression. Bioinformatics analysis revealed a complete HSE on the upstream -851/-165bp of the HMGB1 gene. However, whether HSF1 regulate the HMGB1expression via binding to the HSE of HMGB1

has not been clarified yet. Based on the previous study, we investigated the mechanism for HSF1 regulating the HMGB1 expression.

Materials and methods

Main reagents

The RAW264.7 cell line was provided by the Department of Pathophysiology of Central South University (Changsha, China). HSF1 monoclonal antibody was purchased from Becton, Dickinson and Co., Ltd. (Franklin Lakes, NJ, USA). HMGB1 monoclonal antibody was obtained from UpState Co., Ltd. (New York, USA). Pyrobest enzyme and restriction enzymes were products of TaKaRa Bio. Co., LTD (Dalian, China). Lipofectamine 2000 and pGL3-Basic vector was purchased from Promega and Invitrogen Corporation respectively. Hotstar enzyme, Tip100 plasmid extraction kit, and SYBR-Green PCR Master Mix were obtained from Qiagen Inc. (Montgomery, MD, USA). Dual Luciferase assay kit was purchased from Invitrogen Co., Ltd. (Shanghai, China). Nuclear protein extraction kit was a product of Active Motif Company (BERTEC Co., Ltd, Taiwan, China).

Bioinformatics analysis

Matinspector software was used to analyze the binding sites of transcription factor in the promoter region of HMGB1. Matinspector 2.2 version was used for blast analysis based on the transcription factor binding site database indexed in Transcription Factors Database 7.0. The available website is http://www.genomatix.de/cgi-bin/matinspector_prof/mat_fam.pl.

The construction of recombinant vectors

According to bioinformatics analysis and PCR primer design principle, PrimePrimer 5.0 software was used to design HMGB1 primers and they were synthesized by Boshang Biotechnology Co., Ltd. (Shanghai, China). The promoter region of mouse HMGB1 was amplified and the PCR product was digested by restriction enzymes Xho I /Kpn I followed by a ligation into a PGL3-Basic plasmid. After sequencing, the recombinant plasmid was confirmed and named pGL3-HMGB1-Y, a luciferase reporter gene for the promoter of wild-type HMGB1. Subsequently, over-lap method was employed to amplify the product via introducing mutant bases primers (**Table 1**) based on the template of pGL3-HMGB1-Y. The site-directed mutant fragment was digested by Xho I/Kpn I followed by a ligation into a PGL3-Basic plasmid. After the sequencing, the recombinant plasmid was named pGL3-HMGB1-T, a luciferase reporter gene plasmid for the promoter of mutant HMGB1. The recombinant plasmid was proved to have a consistent promoter region with mouse HMGB1. The plasmid was harvest and extracted by Tip100 and then stored at -20°C for late use.

Cell culture and transient transfection

The transfection procedure was performed in accordance with the manufacturer's instructions. In brief, the RAW264.7 cells were cultured to a confluence of 70%-80%. Two $\times 10^5$ cells were placed in a 6-well plate and incubated in an atmosphere of 5% CO_2 at 37°C for 24 hours. Then the cells were cultured with serum-free RPMI1640. Following, 1 μl of pGL3-IL10 luciferase reporter plasmid and a control plasmid phRL-SV40 was mixed with 8 μl of Lipofeetin transfection reagent respectively. The mixture was then added to the cells and co-cultured for another 12 hours. After that, the cells were collected and lysed.

EMSA assay

Two biotin labeled complementary oligonucleotides containing the HSE (wild or mutant type) DNA fragments were synthesized by Invitrogen Biotechnology Co., Ltd. (Shanghai, China) (**Table 1**). Before adding the labeled probes, 3.0 μl of 5 × binding

Table 1. Primers and probes for recombinant plasmid construction and EMSA assay

Gene	Primer pairs
HMGB1MT-F	5' – GTACGAGCCGTACTGGGGGAAACCCGAGCAAAC – 3'
HMGB1MT-R	5' – TTTCCCCCAGTACGGCTCGTACTTTTTTTTTTTTTTCC – 3'
HSE-F	5' – TCCTAATATGTTCTGGAATAGCCCATT – 3'
HSE-R	5' – AATGGGCTATTCCAGAACATATTAGGA – 3'
HMGB1WT-F	5' – CCAATTCTCGAGGTGTATGGGGGTGGGGCAATGAGGTC – 3'
HMGB1WT-R	5' – GACACAAGCTTTTGGGGATGCCTCCCCGAGTGGG – 3'

buffer and 15 μg (approximately 3-5 μl) of nuclear protein extracts were mixed to reach a total volume of 15.0 μl. For the competitive reaction, 30 ng of unlabeled competitive or noncompetitive oligonucleotide was added in this system. The molar ratio of the competitive probe and the labeled probe was 50:1. The sample was separated on 4% PAGE for 45 minutes at 350 V. The gel was imaged at -70°C after exposure for 24 hours.

Analysis of dual-luciferase activity

The process was done according to the manufacturer's instructions (Promega, USA). In brief, the RAW264.7 cells were lysed and then washed with phosphate buffer twice. 250 μl of reporter gene lysis buffer was added to cover the cells. Then the cells were scraped and then transferred in a 1.5-ml eppendoff tube followed by a centrifuge at 12,000 × g for two minutes. The supernatants were harvest to assay the dual-luciferase activity. 20 μl of RAW264.7 cell lysis products and 100 μl of luciferase substrate were mixed and then the fluorescence value was read. The detection time was 15 seconds and three samples were set for each treatment.

Statistics and presentation of data

All data are expressed as mean±standard deviation. Each experiment was repeated at least three times, and each data represents the mean of at least three parallel samples. SPSS 11.0 statistics software (SPSS Inc., IL, Chicago, USA) was employed to analyze the data. Analysis of variance (ANOVA) was used to compare the differences among groups. A *P* value of less than 0.05 was defined as significant.

Results

Bioinformatics analysis

A complete heat shock element (-TTCGAG AA-) was screened from -851 bp /-165bp region on the upstream of HMGB1 gene (Accession: No.NM_010439) (**Figure 1**). It might be a cis-acting element for HSF1. In this part of the experiment, we amplified the promoter sequence of mouse HMGB1 to construct a luciferase reporter gene of wild-type promoter of HMGB1 on a template of this cis-acting element. In addition, the core base sequence (-T TCGAGAA-) was mutated to construct a luciferase reporter gene of mutant promoter of HMGB1.

Construction and identification of recombinant plasmid

Recombinant pGL3-HMGB1-Y was digested by Xho'!/Hind b!. After electrophoresis, the two fragments including a 4800-bp product (pGL3-basic) and a 429-bp product (Mouse HMGB1 promoter) appeared in the corresponding site respectively (**Figure 2**). The sequencing result for the recombinant plasmid pGL3-HMGB1-Y showed that sub-cloned promoter sequence of mouse HMGB1 was consistent with the sequence screened in Genbank, which suggested a successful construction of the recombinant plasmid pGL3-HMGB1-Y, a luciferase reporter gene of mutant promoter of HMGB1. According to the wild-type HMGB1 promoter sequence and bioinformatics analysis of the HSE, we constructed a luciferase reporter gene of the mutant HMGB1 promoter. The sequence's blasting result showed that the core base -TTCGAGAA- has mutated to -TACGAGCC- (**Figure 3**), suggesting a successful construction of pGL3-HMGB1-T, a luciferase reporter gene of mutant HMGB1 promoter.

851GTGTATGGGGGTGGGGCAATGAGGTCTTTAAAGAACTTGCTAATTCTAGCTGGGCCCCCCCCC
AAACATTTTCTGAGAACAGCCAGTATCAAACCCAGTGGCTGTGCATTAATTCCCTGGTATAAGGCT
TTGGTGTCCGGGAAGTTCAAAACAGTGCTTTGTGTCTCACCAAAAGGAAAAAAAAAAAAAAAGT
TCGAGAAGTACTGGGGGAAACCCGAGCAAACCCAGAAGAAGGGGTGCAACTGGACGCCTGCTGG
CGGGTGCAGACTAGGCTTCTGGGCAGGGTGGGCACGTTAGGCGGGCAGTCCCGGGGAGGGGTGT
CTGGTGGTGGGCTGCAGCAGCACGGCCGTCCCGGAGCCCGCAGAGGCCCCGTGTTTGGGCCACAG
GCCCCCCCGAGCAGTCACCCCACTCGGGGAGGCATCCCCAAGCACGGGCCGCACATCCCAACGGC
TCGGGGCGGGAGCGTGGAGCGGGAGGGGGGGCGGGCCTGCGGGGAGGCGCCCGGGCCTGGGGA
CGCAGCCGTGGCTGCGGCGGAGCCGGGCCCTGGCCGCGCGCCGCGGCTCGGGCGTGGGGCTG
GCTCGGGGCAGGCGAGGCACAGAGACACCATAGAGCTCCGCTTTCCCGTAGCAGCTGCTGCCTCT
GCCGCTGCCTCTCCCGCCTCAGCCTCTCGGCCCGGCACAC-165

Figure 1. Bioinformatics analysis of mouse HMGB1 gene

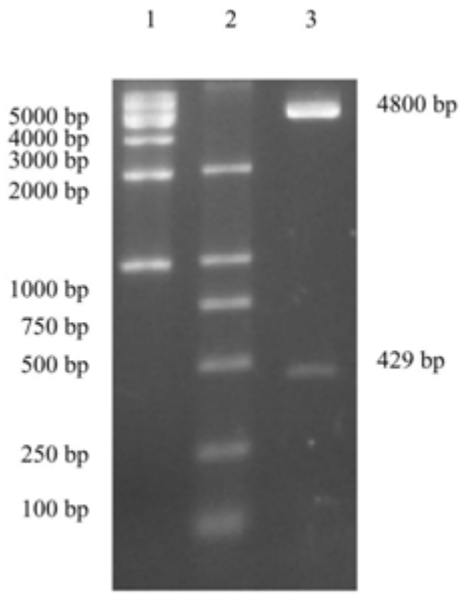

Figure 2. Digestion of the recombinant plasmid pGL3-HMGB1-Y by Xho/Hind III. Lane 1: 1kb DNA Marker; Lane 2: DL2000 DNA Marker; Lane 3: Recombinant pGL3-HMGB1-Y plasmid.

Recombinant pGL3-HMGB1-Y was digested by Xho I /Hind II. After electrophoresis, the two fragments including a 4800-bp product (pGL3-basic) and a 429-bp product (Mouse HMGB1 promoter) appeared in the corresponding site respectively (**Figure 2**). The sequencing result for the recombinant plasmid pGL3-HMGB1-Y showed that sub-cloned promoter sequence of mouse HMGB1 was consistent with the sequence screened in Genbank, which suggested a successful construction of the recombinant plasmid pGL3-HMGB1-Y, a luciferase reporter gene of mutant promoter of HMGB1. According to the wild-type HMGB1 promoter sequence and bioinformatics analysis of the HSE, we constructed a luciferase reporter gene of the mutant HMGB1 promoter. The sequence's blasting result showed that the core base -TTCGAGAA- has mutated to -TACGAGCC- (**Figure 3**), suggesting a successful construction of pGL3-HMGB1-T, a luciferase reporter gene of mutant HMGB1 promoter.

EMSA assay

A retardation strap can be observed after the co-culture of biotin labeled HSF1 binding fragment and nuclear protein extracts. The retardation can be competed by unlabeled probe and not by unlabeled mutant probe. A retardation strap was present after the HSF1 monoclonal antibody adding, suggesting a special binding of HSF1 and HSF1 recognition sequence. In the negative control, no nuclear protein was added. In the HSE-WT, a retardation strap appeared after the nuclear protein adding. No retardation strap was present in HSE-Consensus. A retardation strap was seen after the non-specific competition of AP2. A binding strap was observed after the super retardation by the HSF1 antibody. HSF1 antibody displayed a super retardation effect on -HSF1-wt as can be seen in **Figure 4**.

Double luciferase analysis

When the HSE core base -TTCGAGAA- was mutated to -TACGAGCC-, the relative luciferase value in the plasmid pGL3-HMGB1-T was significantly reduced compared with that in the plasmid wild-type pGL3-HMGB1-Y (ANOVA, F=12.711, P <0.01) (**Table 2**).

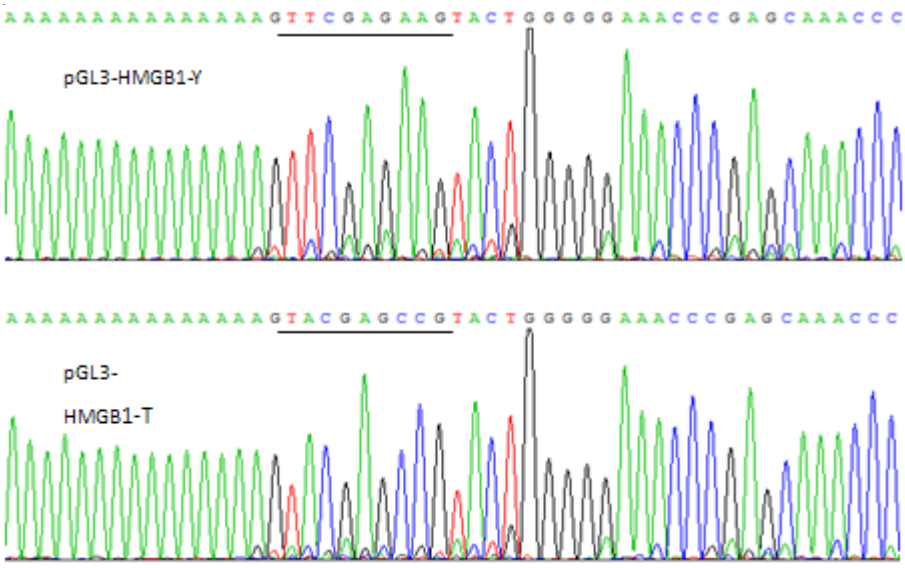

Figure 3. Sequence blast of plasmids pGL3-HMGB1-Y and pGL3-HMGB1-T

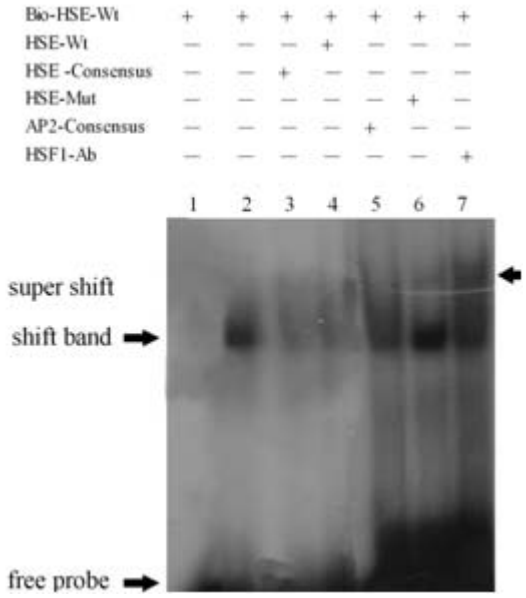

Figure 4. EMSA assay of HSF1 binding to the HSE in the promoter region of HMGB1

Table 2. The relative luciferase activity of the plasmids pGL3-HMGB1-Y and pGL3-HMGB1-T ($\bar{x}$ ±s, n=3)

Plasmid type	Relative luciferase activity
Control	29.62±2.45
Wild	100.25±3.26
Mutant	30.56±1.37

Discussion

HMGB1 is a key signal molecule that can induce immune response to cell damage and death. Normal RAW264.7 macrophages express no HMGB1, whereas burn serum can induce the HMGB1 protein expression in time- and dose dependent manners [5]. HMGB1 is a cytokine with a molecular weight of 18kDa, and it can regulate inflammatory mediators and immune response. It was found that the HMGB1 expression was regulated by transcription factors SP1 and SP3. Further study confirmed a strong role of SP1 in the promoter region of HMGB1 gene in macrophages after the stimulation of LPS [9]. The binding sites of NF-Y/HMG-I (Y) were found in the promoter region of human HMGB1 gene [10, 11]. These findings all suggest that the HMGB1 is carefully regulated to respond to the external stimuli at transcriptional level.

Recently, some scholars have confirmed that HSR could reduce LPS induced TNF-α and HMGB1 expressions via inhibiting phosphorylation activation of p38 and ERK [12]. In addition, the presence of HSF1 gene inhibited LPS induced inflammatory mediators releasing [13]. The binding of HSF1 and HSE inhibited the transcription of TNF-α, IL-1β and IL-15 [14, 15]. The binding initiated iNOS transcription by binding to the HSE, thereby promoting NO synthesis, which displayed an antibacterial property [16]. Meanwhile, the complete HSE sequence (nGAAnnTTCn) was found in inflammatory mediators [17]. Further study showed that HSF1 also could regulate LPS induced IL-1β gene transcription in monocytes via interacting with an important IL-1β gene transcription factor NF-IL6 [18]. The studies suggest that HSF1 can regulate the expressions of inflammatory mediators via directly binding to the promoter region HSE of inflammatory mediators or (and) interacting with the inflammation associated transcription factors.

In the previous work, we investigated the regulatory mechanism of HSF1 for IL-10 transcription of and found that HSF1 could specifically bind to the promoter HSE (-376bp ~-369bp). The relative luciferase activity analysis revealed that HSF1 could activate IL-10 and up-regulated its expression. A complete heat shock element (-TTCGAGAA-) was found in the upstream region (-851bp/-165bp) of the HMGB1 gene after the bioinformatics analysis. In addition, this fragment might be a binding sequence of HSF1, which was also named 'HSF1 recognition sequence'. In order to understand whether the above fragment can bind to the HSE protein, we first synthesized the fragment and amplified the promoter of mouse HMGB1 gene to construct a luciferase reporter gene of wild-type HMGB1 promoter. Then we mutated the core base -TTCGAGAA- and constructed a luciferase reporter gene of mutant HMGB1 promoter. Subsequently, EMSA was employed to assay the binding of HSF1 and the wild and mutant type promoter respectively.

The DNA binding activity of the transcription factor can be assayed *in vitro* and *in vivo*. *In vivo* the interactions between the transcription factors and the nucleic acids can be studied by electrophoretic mobility shift assay (EMSA). The interactions between protein sequences and DNA sequences, also the interactions between DNA binding proteins and the related DNA binding sequences, can be used for qualitative and quantitative analysis. The reaction specificity can be verified by antibodies, peptides, or mutant oligonucleotides. In the EMSA experiment, a competitive experiment was done by HSE site-directed mutation method. The result showed that a retardation strap could be observed after the co-culture of biotin labeled HSF1 binding fragment and nuclear protein extracts. The retardation can be competed by unlabeled probe not by unlabeled mutant probe. A retardation strap was present after the HSF1 monoclonal antibody adding, suggesting a special binding of HSF1 and HSF1 recognition sequence.

In the present study, we linked a luciferase reporter gene to response elements, which could selectively assay the expression products via measuring signaling cascades activity. To preliminarily investigate the effects of HSF1 on the transcription of HMGB1, we constructed a mutant pGL3-HMGB1-T of a core base of 'HSF1 recognition sequence' and compared the differences in the transcriptional activity between them and the recombinant plasmid wild-type pGL3-HMGB1-Y. When the HSE core base -TTCGAGAA- was mutated to -TACGAGCC-, the relative luciferase value of the mutant pGL3-HMGB1-T was significantly decreased compared with that of wild-type pGL3-HMGB1-Y. In addition, the mutation of the HSE core base down-regulated the transcriptional activity of HMGB1. Our results suggest HSF1 can down-regulate the HMGB1 expression at transcriptional level. And, we also found the existence of a negative feedback pathway between HSF1 and HMGB1, which provided a new understanding of the regulatory mechanism for the HMGB1 and HSF1 controlling cytokines' expressions.

In summary, both of the most important late inflammatory mediator HMGB1 and anti-inflammatory mediator IL-10 are regulated by the HSF1 gene. Importantly, there are HSF1 binding sites in the promoter region (-851bp ~-165bp) of the HMGB1 gene. Further study is needed to investigate the mechanism for HSF1 anti-inflammatory effect and endogenous cellular protection in the inflammatory reaction after burn.

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