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Heparin changes the conformation of high-mobility group protein 1 and decreases its affinity toward receptor for advanced glycation endproducts *in vitro*

Yan Ling, Zhi-Yong Yang, Tao Yin, Li Li, Wei-Wei Yuan, He-Shui Wu, Chun-You Wang *

Pancreatic Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei Province 430022, China

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ABSTRACT

High-mobility group protein 1 (HMGB1) has been identified as a late-acting mediator of inflammation. The receptor for advanced glycation end products (RAGE) is the main receptor and mediates the cytokine activity of HMGB1. Since HMGB1 also exhibits heparin-binding activity, we investigated whether heparin interferes with HMGB1/RAGE interaction and prevents the cytokine activity. We used fluorescence spectrometry, circular dichroism spectrometry and SPR biosensor technique to evaluate the effect. After treatment of HMGB1 with different concentrations of heparin (0, 50, 100 and 1000 U/L), the fluorescence peak values of HMGB1 increased and the emission wavelength showed red shifts; further, the secondary structure of HMGB1 showed a marked change in that the content of β-pleated sheet reduced while that of α-helix increased. The equilibrium dissociation constants (K_D) were determined by SPR technique; $K_D = 4.5 \times 10^{-9}$ mol/L for heparin and HMGB1 and $K_D = 9.77 \times 10^{-8}$ mol/L for HMGB1 and RAGE, respectively. Heparin and RAGE had no interaction. The amount of HMGB1 and RAGE bound forms reduced after treatment with heparin. ELISA revealed that addition of heparin inhibited the TNF-α and IL-6 released by macrophages RAW264.7 and HUVEC; 10 U/L and 50 U/L of heparin showed the most marked inhibitory effect in RAW264.7 cells and in HUVEC, respectively. In conclusion, heparin can combine with HMGB1 and affect the affinity of HMGB1/RAGE by changing the conformation of HMGB1. And this effect was independent of heparin concentration, so that a low dose of heparin was sufficient to achieve the best anti-inflammatory effect in our test.

1. Introduction

Sepses, diabetes, rheumatic arthritis, sever acute pancreatitis and so on are common, and are high risk for human health. And the systemic inflammatory response syndrome (SIRS) occurred during the diseases is still a problem that disturbs the treatment and the high-mobility group protein 1 (HMGB1) was found playing a vital role in the progress.

The high-mobility group protein 1 (HMGB1), which is also known as P30, amphoterin, or HMG-1, is a highly conserved protein and shows above 99% amino acid homology between HMGB1 of human beings and that of rodents [1]. This protein has a highly dipolar structure: it contains a 185-amino acid basic region and a 30-amino acid cluster of acidic residues at the C-terminus; the band of this protein migrates to the 30 kDa position on a standard polyacrylamide electrophoresis gel. The 185-amino acid basic part is subdivided into 2 homologous HMG boxes, namely, box A and box B, each around 75 amino acids in length. Both the boxes have a similar α-helical structure to allow DNA binding [2,3]. The three-dimensional structure of the whole HMGB1 molecule has not been elucidated to date. The N-terminus of HMGB1 (residues 6–12) contains a consensus sequence that has also been identified in several heparin-binding proteins [4], which likely contributes to the heparin-binding ability of HMGB1. The region between the HMG-box B and the acidic tail of HMGB1 (residues 151–183) is the most distinctive feature of HMGB1 in comparison with the characteristics of the other members of the HMG protein family. This region of HMGB1 contains a motif that can bind to receptor for advanced glycation end products (RAGE) [5]. The highly acidic C-terminus of HMGB1 plays an important role in nuclear functions [6,7]. The proinflammatory activity of HMGB1 is localized to the HMG-box B, especially to the residues 89–108 [8].

HMGB1 is a nonhistone DNA-binding protein; it is involved in stabilizing nucleosome formation, increasing gene transcription, and modulating steroid hormone receptors [9,10]. Recently, HMGB1 has been identified as the late-acting mediator of endotoxin lethality [11]. When released from cells, HMGB1 can bind to cell-surface receptors [e.g., RAGE, Toll-like receptor (TLR) (TLR2 and TLR4)] and mediate the chemotactic cell movement and the release of proinflammatory cytokines [e.g., tumor necrosis factor (TNF) and interleukin (IL)-1] [12,13].

RAGE is expressed in endothelial cells, vascular smooth muscle cells, neurons, and macrophages/monocytes [14] and it is the main HMGB1 receptor. RAGE is a 50 kDa protein (404 amino acids in the protein sequence of humans) and belongs to a member of the immunoglobulin superfamily of cell-surface molecules. This protein is composed of an extracellular region containing 1 V-type immunoglobulin (Ig) domain

* Corresponding author.
E-mail addresses: lingyan1029@163.com (Y. Ling), chunyouwang52@126.com (C.-Y. Wang).

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and 2 C-type Ig domains; a hydrophobic transmembrane-spanning domain; and a highly charged short cytoplasmic domain [15]. The ligand-binding properties of RAGE can be attributed to the distal V-type domain that contains 2 potential N-glycosylation sites [16].

Heparin, a highly sulfated glycosaminoglycan, has been used as an anticoagulant for clinical purposes for more than 70 years. It has been recently discovered that heparin possesses anti-inflammatory activity; however, the mechanisms of this activity remain to be determined. In our previous research, we have found that heparin inhibits the proinflammatory activity of HMGB1. Heparin interferes with the specific binding of HMGB1 with RAGE by causing conformation changes in HMGB1. In this study, we evaluated the effect of heparin on HMGB1 and investigated the underlying mechanisms. Here, we provided a novel therapeutic method and an evidence for using heparin and its analogues in treatment of inflammatory reaction.

2. Materials and methods

2.1. Equipment and reagents

2.1.1. Equipment

Fluorescence spectrometer FP-6500 and circular dichroism (CD) spectropolarimeter J-810 were from JASCO (Tokyo, Japan); surface plasmon resonance (SPR) measurements were performed on a BIAcore 3000 system from BiacoreAB (Uppsala, Sweden) by using the BIAcore evaluation software version 4.0.

2.1.2. Reagents

HMGB1 and heparin were purchased from Sigma-Aldrich (USA); RAGE (membrane bound derivative) was purchased from R&D systems (USA). Fluorescence spectrometry and CD spectropolarimetry analysis were performed using 0.05 M Tris–HCl buffer (pH 7.4) containing 0.10 M NaCl to maintain the ionic strength. The BIAcore research-grade sensor chip CM5 (composed of carboxymethylated dextran coupled to gold-coated glass surface and prepared according to procedures described elsewhere), amine-coupling kit [containing *N*-ethyl-*N*-(dimethyaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), and a solution of 1 M ethanol amine hydrochloride whose pH was adjusted to 8.5 using sodium hydroxide], and HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% P20; pH 7.4) were purchased from BiocoreAB (Uppsala, Sweden). Medium 1640 was purchased from Hyclone (USA). ECM (endothelial cell medium) was purchased from Sciencell (USA). All solutions were filtered through 0.22-μm Watman filters and degassed (Milipore sintered glass funnel) before use. All experiments were conducted at 25 °C.

2.2. Experimental methods

2.2.1. Fluorescence spectroscopy and synchronous fluorescence spectroscopy analysis of HMGB1

Steady-state fluorescence measurements were obtained using a FP-6500 spectrophotometer equipped with a 0.1-cm quartz cell. HMGB1 (0.025 g/L) was mixed with different heparin concentrations of 0, 50, 100, and 1000 U/L. The samples were incubated at 25 °C for 15 min before further analysis. The excitation wavelength was 280 nm, the width of the excitation and emission slits was 3 nm, and the scanning speed was 500 nm/min. Synchronous scanning fluorescence spectra were recorded as described by Rao [17]. The excitation and the emission wavelengths (λ_{ex} and λ_{em}) for synchronous fluorescence measurements were scanned simultaneously with a constant wavelength interval ($\Delta\lambda = 15$ nm and $\Delta\lambda = 60$ nm) between λ_{ex} and λ_{em} and with the speed of 500 nm/min.

2.2.2. CD spectroscopy of HMGB1

The wavelengths in the CD spectrum (range, 190–250 nm) were recorded using a quartz cuvette with a path length of 10 mm. The scanning speed was 1000 nm/min. The samples were prepared according to the abovementioned method. Each sample was analyzed 3 times, and the conformation of the unit was calculated according to Chen–Yang's method [18].

2.2.3. Immobilization of HMGB1 and RAGE on CM5 sensor chip surface

Standard BIAcore amine-coupling protocol involving EDC and NHS was applied. HMGB1 was diluted with 10 nM sodium acetate (pH 5.0) to the concentration of 50 mg/L, and RAGE was diluted with 10 nM sodium acetate (pH 4.5) to the concentration of 40 mg/L. The concentrations of stock EDC/NHS solutions were 0.2 M and 0.05 M, respectively. The flow cells (Fc) 2 and Fc4 contained immobilized HMGB1 and RAGE, respectively, while Fc1 and Fc3 were used as negative controls.

2.2.4. Analysis of heparin–HMGB1 and HMGB1–RAGE interactions

Heparin samples of different concentrations were diluted with HBS-EP and 20-μL aliquots of this solution were injected over Fc2 on CM5 sensor chip at a flow rate of 30 μL/min. Next, HMGB1 samples of different concentrations were diluted with HBS-EP and 20-μL aliquots were injected over Fc4 at a flow rate of 30 μL/min. At the end of injection, HBS-EP buffer was allowed to flow freely over the sensor surface to facilitate disassociation. After a 2-min dissociation time, the sensor surface was regenerated by injecting 10 mM NaOH. Each cycle included a 2 min break time to allow the monitoring of baseline stability. All Biacore data were collected at pH 7.4 by using HBS-EP as the running buffer and a constant flow rate of 30 μL/min.

2.2.5. SPR binding analysis

HMGB1 diluted with 50 mg/L HBS-EP was injected over the chip surface for 3 min; after a 150-s dissociation period, HMGB1 plus heparin (50, 100, 1000, and 10,000 U/L) solutions were injected over the chip surface for 3 min in the given order of concentrations. Then, the chip surface was regenerated by injecting 10 mM NaOH. All data were collected with running buffer and at a constant flow rate of 20 μL/min.

2.2.6. SPR data analysis

When performing binding assays for heparin–HMGB1 and HMGB1–RAGE by using the BIAcore 3000 system, the binding-response signals were continuously recorded in response units (RU) and presented graphically as the function of time. The BIAcore 3000 system uses a flow-injection system and allows the running buffer to flow over the chip surface at a constant flow rate. Hence, it could be presumed that the concentrations of the samples were constant. Therefore, we adopted the 1:1 (Langmuir) binding fitting model in which the association (ka) and dissociation (kd) constants are fitted simultaneously using Eq. (1) [19]:

$$dR/dt = ka.C(R_{max}-R)-kdR, \quad (1)$$

where R represents the response unit; C is the concentration of ligand (heparin or HMGB1); and R_{max} stands for the maximal response. The affinity constant (K_A) and the equilibrium dissociation constant (K_D) could be obtained from ka and kd by using the following relations:

$$K_A = ka/kd \quad K_D = kd/ka. \quad (2)$$

2.2.7. Detection of TNF-α and IL-6 in cell supernatant

Intact RAW 264.7 macrophages were added to medium 1640 (10% fetal calf serum or FCS) and cultured in 24-hole plate at a density of 4×10^4 in 37 °C and 5% CO_2 for 24 h; subsequently, this medium was replaced by serum-free opti-MEM medium and the cells were incubated for another 24 h. The cells were stimulated with HMGB1 (0.1 μg/mL) and different concentrations of heparin (0.1 U/L, 1 U/L, and 10 U/L) at