Two immunoregulatory peptides with antioxidant activity from tick salivary glands

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Running head: Tick immunoregulatory peptides

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Ticks are blood-feeding arthropods that may secrete immunosuppressant molecules, which inhibit host inflammatory and immune responses and provide survival advantages to pathogens at tick bleeding sites in hosts. In current work, two families of immunoregulatory peptides, hyalomin-A and -B were firstly identified from salivary glands of hard tick \textit{Hyalomma asiaticum asiaticum}. Three copies of them are encoded by identical gene and released from the same protein precursor. Both hyalomin-A and -B can exert significant anti-inflammatory functions, either by directly inhibiting the host’s secretion of inflammatory factors such as TNF-alpha, MCP-1 and IFN-gamma, or by indirectly increasing the secretion of immunosuppressant cytokine of IL-10. Hyalomin-A and -B were both found to potently scavenge free radical \textit{in vitro} in a rapid manner, and inhibited adjuvant-induced inflammation in mouse models \textit{in vivo}. The JNK/SAPK subgroup of MAPKs signaling pathway was involved in such immunoregulatory functions of hyalomin-A and -B. These results showed that immunoregulatory peptides of tick salivary glands suppress host inflammatory response by modulating cytokine secretion and detoxifying reactive oxygen species.

Ticks are parasites that feed on the
blood of their hosts. Their salivary glands can secrete various immunomodulatory molecules to inhibit host inflammatory and immune responses (1-7). Ticks are second only to mosquitoes as vectors of disease-causing agents to humans, and the most important arthropod capable of transmitting pathogens to other animal species (8-13). Medically important tick-borne diseases include Lyme disease (14, 15), tick-borne encephalitis, granulocytic ehrlichiosis (16-19), babesiosis (2, 7, 12), and Crimean–Congo hemorrhagic fever which occurs sporadically throughout much of Africa, Asia and Europe and results in an approximately 30% fatality rate (16-19).

Many reports have showed that proteins extracted from saliva and salivary glands of several ticks may inhibit humoral immunity and the B- and T-cell responses to tick-transmitted pathogens (1, 20-26), and furthermore, may facilitate the pathogen transmission and infection to their hosts by downregulating host immunity (27-34), altering blood flow (30, 35, 36) and inhibiting inflammation (37-41). For example, tick saliva-enhanced transmission has also been demonstrated for several viral and bacterial pathogens, including tick-borne encephalitis virus and Borrelia burgdorferi spp., the causative agent of lyme disease (11, 22, 38). It has been suggested that anti-inflammation mechanism is well conserved among tick species although the presence of marked molecular polymorphism in the protein profile of salivary glands from individual ticks (11). Many literatures have shown that saliva and salivary gland extracts of ticks can inhibit host inflammatory responses by modulating its cytokine secretion or directly block cytokines’ activities via extracts-cytokine interactions (1, 20, 24, 26, 27, 32). Some cytokine-binding peptides and proteins have been identified from tick salivary glands, but so far no regulatory peptides affecting cytokine secretions have ever been characterized (27, 39-41). In current work, two families of immunoregulatory peptides, hyalomin-A and -B were identified from salivary glands of hard tick H. asiaticum. Hyalomin-A and -B were shown to suppress host inflammatory response by modulating cytokine secretion and detoxifying reactive oxygen species.

**Experimental Procedures**

*Salivary gland dissection*- Partially fed adult hard ticks (H. asiaticum) of both sexes (n = 2300) were collected from Xinjiang Province of China (May to July, detached from camels). Ticks were glued to the bottom of a Petri dish and placed on ice for 20 min. They were then incised along the dorsal-lateral margin, and the dorsal integument was removed. The salivary gland was isolated and transferred into 0.1 M phosphate buffer solution (PBS) containing protease inhibitor cocktail (Sigma, P2714), pH 6.0, and kept at -20°C.

*Peptide purification*- The salivary glands from ticks were homogenized using a glass homogenizer in 0.1 M PBS, pH 6.0, containing protease inhibitor cocktail. The homogenate was centrifuged at 10000 rpm. The supernatant (10 ml) was applied to a Sephadex G-50 (Superfine, Amersham...
Biosciences, 2.6 × 100 cm) gel filtration column equilibrated with 0.1 M PBS, pH 6.0. Elution was performed with the same buffer, collecting fractions of 3.0 ml while the absorbance was monitored at 280 nm. The effects of eluted fractions on interferon-gamma (IFN-γ) secretion were determined as described below. The fractions with desired activity was pooled (about 20 ml), lyophilized, re-suspended in 2 ml 0.1 M PBS, and purified further by C18 RP-HPLC (Hypersil BDS C18, 30 × 0.46 cm) as illustrated in Fig. S1B.

Structural analysis- Purified peptide were subject to the complete peptide sequencing by Edman degradation on an Applied Biosystems pulsed liquid-phase sequencer, model 491. Mass spectrometry analysis was performed by using a Matrix-Assisted Laser Desorption Ionization Time-Of-Flight mass spectrometer (MALDI-TOF-MS) AXIMA CFR (Kratos Analytical) in positive ion and linear mode. The operating parameters were as follows: the ion acceleration voltage was 20 kV, the accumulating time of single scanning was 50 s, using polypeptide mass standard (Kratos Analytical) as external standard. The accuracy of mass determinations was within 0.01%.

Construction and screening of cDNA library- mRNAs were prepared from the total RNA of H. asiaticum salivary glands by oligo(dT) cellulose chromatography. A cDNA library was constructed using a SMART™ PCR cDNA synthesis kit (Clontech, Palo Alto, CA) as described (34). The first strand was synthesized by using cDNA 3’ SMART CDS Primer II A, and SMART II oligonucleotide provided by the kit. The second strand was amplified using Advantage polymerase by 5’ PCR primer II A provided by kit. All the PCR conditions are according to the manufacture instruction. A directional cDNA library was constructed with a plasmid cloning kit (SuperScript™ Plasmid System, GIBCO/BRL) according to the instructions of the manufacturer, producing a library of about 2.3 × 10^5 independent colonies.

Two pairs of oligonucleotide primers, S1 (5’-CARACNCCNXGNACNATNGGN-3’, in the sense direction, where N is A, T, C, or G; R is A or G and X is A or C)/Primer IIA (in the antisense direction) and S2 (5’-ACNCTNXGNACNACNGAY-3’, in the sense direction, where Y is T or C)/ Primer IIA (in the antisense direction) were used to screen hyalomin-A and –B, respectively. S1 and S2 are specific primers designed according to the amino acid sequence determined by Edman degradation. All the oligonucleotide primers for PCR were prepared with a DNA synthesizer (Model 381A, Applied Biosystems). The PCR conditions were: 2 min at 94°C, followed by 30 cycles of 10 sec at 92°C, 30 sec at 50°C, 40 sec at 72°C. DNA sequencing was performed on an Applied Biosystems DNA sequencer, model ABI PRISM 377.

Stimulation of rat splenocytes and cytokines assays- A suspension of splenocytes from Wistar rats in RPMI 1640 medium (Gibco Life Technologies) supplemented with 5% fetal bovine serum and 100 U/ml penicillin and streptomycin, were seeded to the wells.
of a 96-well plate. A total of $6 \times 10^6$ cells (100 µl per well) were incubated at 37°C and 3.5% CO$_2$. 20 µl samples dissolved in RPMI 1640 medium with different concentration and lipopolysaccharide (final concentration of 2 µg/ml, Sigma) were co-cultured with splenocytes. Supernatants of the cultures were harvested after 48 h cultivation and stored at -70°C. All combinations were set up in triplicate. Cytokines assays for IL-10, IFN-γ and MCP-1 were detected using antibody-sandwich enzyme-linked immunosorbent assays (ELISAs) using the kits from Adlitteram Diagnostic Laboratories, Inc, USA according the manufacture instruction.

**Free radical scavenging activity**- Free radical scavenging activity of hyalomin-A1/-B was assayed by measuring the reduction of cationic radical 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS$^+$) according to manufacture instruction of the kit GMS10114.4 (Genmed Scientifics INC, Shanghai Branch). The total formation of products (i.e. the reduced form of ABTS and the purple hyalomin-A1/-B modification) and the total consumption of ABTS radical during the initial, rapid reaction phase were determined by linear regression analysis. The concentrations of ABTS and ABTS$^+$ were calculated by using $\varepsilon_{340} = 4.8 \times 10^4$ M$^{-1}$ cm$^{-1}$ and $\varepsilon_{415} = 3.6 \times 10^4$ M$^{-1}$ cm$^{-1}$, respectively (18, 19). The purple hyalomin-A1/-B modification was monitored at 550 nm (18). The purple end product of hyalomin-A1/-B with ABTS$^+$ was purified by a Sephadex G-25 Fine column (Volume, 30 x 1.6 cm) eluted with 2 mM NH$_4$HCO$_3$, pH 8.5 at a flow rate of 0.3 ml/min, and 0.3-ml fractions were collected manually (Fig. S2). The absorbances at 280 nm, 340 nm, 415 nm and 550 nm were monitored. The fraction containing purple hyalomin-A1/-B modification was purified further by RP-HPLC using C$_18$ column and then subject to MALDI-TOF-MS analysis.

**Effects of hyalomin-A1/-B on proliferation of mouse splenocytes in vitro**- Splenocytes from Kunming mouse were harvested and cultured in RPMI-1640 medium supplemented with 5% fetal calf serum, and $1.5 \times 10^6$ cells per 0.2 ml were plated in 96-well plate. Tested sample hyalomin-A1/-B was added at final concentrations of 5, 10, 20, 40, and 80 µg/ml in the presence of 4 µg/ml ConA. After a 44 h incubation at 37°C in a humidified 5% CO$_2$ atmosphere, 20 µl of MTT (3-(4-5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (5 mg/ml) was added to each well for a 4 h incubation. Finally, each well was added a 100 µl of acidified isopropylalcohol, and the cells were homogenized for at least 10 min to fully dissolve the colored material. The absorbance at 570 nm was measured in an ELISA reader.

**Macrophage cell proliferation and viability measurement**- Raw 264.7 murine macrophage cells (RMMCs) were cultured in Dulbecco's Modified Eagle Media (DMEM, 11960-044, Gibco, USA) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 U/ml of streptomycin in a humidified 5% CO$_2$ atmosphere at 37°C. $2 \times 10^4$ RMMCs per well (180 µl) were plated into a 96-well plate. After
overnight incubation, RMMCs were adhered to the plate, then 20 μl of tested samples dissolved in DMEM were added to the wells for 20 h and 44 h incubation, using the same volume of DMEM as blank control. At the end of incubation, 20 μl of MTT solution (5 mg/ml) was added to each well and the cells were further incubated for 4 h at 37 °C. Cells were solved in 200 μl of DMSO, the absorbance at 570 nm was measured in an ELISA reader. The viability of the treated group was expressed as the percentage of control group which was assumed to be 100%.

Western blot analysis- RMMCs (1×10⁶/well) were plated and adhered to a 24-well culture plate. Cells were then transferred to serum-free DMEM for an 18 h-incubation. The cells were pre-treated by peptide samples of various concentrations (2, 4, 8 μg/ml) or blank for 1 h before the addition of LPS (1 μg/ml). After incubation for 15 min, cells were collected by centrifugation and washed twice with ice-cold PBS. The washed cell pellets were resuspended in 150 μl of extraction lysis buffer (50 mM Tris-HCl, pH 7.4; 1% Nonidet P-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 μg/ml each of aprotinin, leupeptin, and pepstatin; 1 mM sodium orthovanadate and 1 mM NaF) and incubated for 30 min at 4°C. Cell debris was removed by centrifugation, followed by quick freezing of the supernatants. The protein concentration was determined using Bradford protein assay. Forty micrograms of cellular protein from treated and untreated cell extracts were separated on a 12% SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto a PVDF membrane. The immunoblot was incubated with blocking solution (5% skim milk) at room temperature for 3 h, followed by incubation overnight with a primary antibody against the phosphorylated forms of Erk1/2, SAPK/JNK, or p38 MAP kinase at 4°C, respectively. Blots were washed three times with Tween 20/Tris-buffered saline (TBST) and incubated with a 1:1000 dilution of horseradish peroxidase-conjugated secondary antibody for 1h at room temperature. Blots were again washed three times with TBST and then developed by enhanced chemiluminescence (Tiangen Biotech).

For the western blotting of pc-Jun, p-ATF-2 and PCNA (proliferating cell nuclear antigen), cellular nuclear extracts were prepared as described below. Cells were washed twice with ice-cold PBS and resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1.5 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF and 1 mM Na₃VO₄). After the addition of 0.5% (v/v) Nonidet P-40 and vigorous vortexing for 10 s, the nuclei were pelleted by centrifugation (12,000 × g for 1min at 4 °C). The collected nuclei pellet was resuspended in extraction buffer (20 mM HEPES, pH 7.9; 400 mM NaCl, 1mM EGTA, 1mM EDTA,1 mM dithiothreitol, 1 mM NaF and 1mM Na₃VO₄). After centrifugation (12,000 ×g for 10 min at 4°C), the supernatant was collected and stored at -70 °C. Twenty micrograms of cellular protein from treated and untreated cell
extracts was electroblotted onto a PVDF membrane following separation on a 12% SDS-PAGE. Procedure of the immunoblotting was the same as above.

Effects of hyalomin-A1/B1 on adjuvant-induced arthritis in mice- 20 μl of vehicle (saline) or Freund’s complete adjuvant (Sigma) was administered into the plantar surface of the right hind paw of male Kunming mice (12/group). The basal footpad thickness of each mouse was measured with a vernier caliper at the beginning of the experiment. Hyalomin-A1/B1 (1.25, 2.5 and 5 mg/kg) were administered into the muscle of hind legs root alternatively from day 1 after injection of Freund’s complete adjuvant to day 14. Control groups of mice received the same volume of saline (vehicle). As an index of inflammation, right hind paw thickness was measured with a vernier caliper at day 1, 5, 9, 11, 15 and 21, respectively.

The experimental protocols were approved by the Animal Care and Use Committee at Kunming Institute of Zoology, Chinese Academy of Sciences.

Peptide synthesis- Hyalomin-A1 and –B1 were synthesized by solid phase synthesis on an Applied Biosystems model 433A peptide synthesizer. After the cleavage and deprotection of side-chain, the crude synthetic peptide was purified on a Vydac C18 RP-HPLC column (25 cm × 1 cm), eluting at a flow rate of 1 ml/min by a linear gradient of acetonitrile in 0.1% trifluoroacetic acid in water. Identity of the peptide was confirmed by automated Edman degradation with a protein sequencer and mass spectrometry analysis.

Statistics- Data analysis was performed using the Statistical Package for Social Science (SPSS 11.5). Statistical analysis data was presented as mean ± standard deviation (SD).

RESULTS

Purification and structural analysis of peptides from tick salivary gland extracts. The crude extracts from the tick salivary glands have been divided into several factions by Sephadex G-50 gel filtration and the fraction indicated by a bar was found to inhibit IFN-γ secretion as illustrated in Fig. S1A. The sample with IFN-γ secretion-inhibitory activity was pooled and further purified by C18 RP-HPLC. Two peptides were purified from this step as indicated 1 and 2 in Fig. S1B. They are named hyalomin-A1 and -B1, respectively. The complete amino acid sequences by Edman degradation of hyalomin-A1 and -B1 were determined as QTPRTIGPPYT and TLRTTTGYWTVEKGNGTTPAANS TEKGNRPYGR, respectively. The observed [M+H]+ from MALDI-TOF-MS were 1231.19 and 3688.27 (Fig. S3), which matched well with the calculated [M+H]+ of 1231.39 and 3688.00, respectively. The amino acid sequences of hyalomin-A1 and -B1 were further confirmed by the cDNA cloning as described below.

cDNA cloning. As illustrated in Fig. 1A, a cDNA of 801 bp (GenBank accession GU828034) was cloned from the cDNA library of salivary gland of tick H. asiaticum asiaticum. This cDNA
encodes a precursor protein composed of 221 amino acid residues (aa). It was found that both hyalomin-A1 and -B1 share the same 221 aa precursor protein. In the sequences of the 221 aa precursor, there are 3 copies of hyalomin-A1 and 3 homologues of hyalomin-B1 (B1 to –B3, Fig 1B). Hyalomin-A1 is composed of 11 amino acid residues. Both hyalomin-B1 and –B2 are composed of 34 residues while hyalomin-B3 is composed of 32 residues. All the sequences of mature hyalomin-A and B peptides are flanked by the possible enzymatic processing site of –RR- (Fig. 1A).

**Effects on cytokine secretion.** We tested the effects of synthesized hyalomin-A1 and -B1 on IL-10, IFN-γ, MCP-1 and TNF-α secretion induced by lipopolysaccharide (LPS) in mouse splenocytes. Four concentrations (0, 2, 4 and 8 µg/ml) of hyalomin-A1 and -B1 were used. LPS alone could induce IL-10, IFN-γ, and MCP-1 secretion. As illustrated in Fig. 2, both hyalomin-A1 and -B1 could markedly inhibit secretions of IFN-γ, MCP-1 and TNF-α induced by LPS in a dose-dependent manner (Fig. 2A, 2C and 2D). The secretion of IL-10 could be significantly increased by hyalomin-B1. Hyalomin-A1 only could slightly increase the IL-10 secretion even at the concentration of 8 µg/ml (Fig. 2B).

**Free radical scavenging activity.** Owing to the good performance with respect to the stability, maneuverability and reproducibility, ABTS+ radical scavenging assay was commonly used to evaluate antioxidant capacity of biomolecules (33). The decrease in absorbance at 734 nm was monitored at 1 min intervals for 30 min. The absorbance of control and sample were monitored simultaneously. Both hyalomin-A1 and -B1 could react with ABTS+ and convert it into colorless compounds. As illustrated in Fig. 3, they could rapidly scavenge ABTS+ in a time-dependent manner. The overlaid scans of the solution were performed to monitor the decrease of the ABTS radical-specific 415 and 735 nm peaks and a concomitant increase of the ABTS-specific peak at 340 nm. Although the ABTS+ displayed a slender auto-scavenging activity (Fig. 3A), both hyalomin-A1 and -B1 could significantly catalyze this process (Fig. 3C and 3D), and the catalysis rate is even much faster than that of commerical antioxidant factor (BHT) (Fig. 3B). The reduction processes by hyalomin-A1 and -B1 were a two-phase reaction. An initial faster phase over several seconds was followed by a second slower phase that was still ongoing after 10 min (Fig. 3A to 3D). Most of the ABTS radical was reduced after 5 s. Two end products of reduced ABTS were found, represented by the 340 nm peak and a novel peak at 550 nm (Fig. 3C and 3D, *inset*). No product with the absorbance at 550 nm was found in the BHT-ABTS react system (Fig. 3B), indicating that BHT might have a different radical scavenging mechanism from these tick peptides.

It has been proved that phenols can react with ABTS radicals to form purple compounds with a broad absorbance around 550nm (32, 39, 40). Among the essential amino acids, only tyrosine contains phenol side chain. One and two tyrosine residues are found in
hyalomin-A1 (Y₁₀) and -B1 (Y₈ and Y₃₂), respectively, and they possibly react with ABTS radicals to form hyalomin-A1/-B1-ABTS purple adducts. The observed masses of hyalomin-A1 and -B1-ABTS purple adducts were 1498.1 and 3954.9, respectively, which were two mass units less than the total masses of hyalomin-A1/-B1 plus ABTS free radical (1500.2 and 3956.8). The two mass units account for the oxidized tyrosine residue as confirmed by previous report (32). As listed in Table S1, Y₁₀ and Y₈ replacement in hyalomin-A1 and -B1 extremely decreased their antioxidant capabilities, whereas Y₃₂ replacement in hyalomin-B1 had only a slight effect on the antioxidant capability, suggesting that Y₃₂ did not take part in the reaction with ABTS radicals.

**Effects on cell proliferation and cell viability.** The effects of hyalomin-A1 and -B1 on cell proliferation were tested by co-culturing with mouse splenocytes in RPMI-1640 medium supplemented with 5% fetal calf serum (Fig. S2). Five sample concentrations (5, 10, 20, 40, 80 µg/ml) were used. Interestingly, all five concentrations of hyalomin-A1 could slightly (about 20%) inhibit splenocyte proliferation in a dose-independent manner. Regarding the hyalomin-B1, low concentrations (5, 10 and 20 µg/ml) showed the same cell proliferation-inhibitory effects as hyalomin-A1 while high concentrations (40 and 80 µg/ml) had no effect on splenocyte proliferation (Fig. S2A). The effects of hyalomin-A1 and -B1 on cell viability were assayed by using Raw264.7 murine macrophages grown in DMEM supplemented with 10% fetal bovine serum and antibiotics. All the tested concentrations (10, 20, 40, 80 and 160 µg/ml) of hyalomin-A1 and low concentrations (10 and 20 µg/ml) of hyalomin-B1 have little effects on the cell viability but high concentrations (40, 80 and 160 µg/ml) of hyalomin-B1 could increase the cell viability by 10-20% (Fig. S2B).

**Influence on MAPK signaling pathway.** The mitogen-activated protein kinases (MAPK) play important roles in the regulation of cell growth and differentiation, and as well the control of cellular responses to proinflammatory cytokines and environmental stresses. The effects of hyalomin-A1 and -B1 on the LPS-stimulated phosphorylations of ERK1/2, SAPK/JNK and p38 MAP kinases in RAW 264.7 macrophage cells were examined using western immunoblot analysis. As shown in Fig. 4, both hyalomin-A1 and -B1 inhibited LPS-induced degradation of IκB-α. Hyalomin-A1 could inhibit the LPS-induced activation of p38 MAP kinase while hyalomin-B1 had no obvious effects on p38 MAP kinase. Neither hyalomin-A1 nor -B1 had marked effect on the LPS-induced activation of ERK1/2 MAP kinase, however, with respect to the LPS-induced activation of JNK MAP kinase, both hyalomin-A1 and -B1 indeed showed significant suppression in a dose-dependent manner. The phosphorylations of JNK1 (p46) and JNK2 (p54) were blocked by both hyalomin-A1 and -B1, and especially, the LPS-induced phosphorylation of JNK1 could be completely blocked at the sample concentrations above 8 µg/ml. To further confirm whether
hyalomin-A1/-B1 mainly act on JNK/SAPK signaling pathway, we examined the effects of hyalomin-A1 and -B1 on upstream and downstream kinases of JNK. It was found that the phosphorylations of M KK4 (the upstream kinase of JNK), C-jun and ATF-2 were all inhibited by hyalomin-A1/-B1 in a dose-dependent manner.

Inhibition of adjuvant-induced inflammation in mouse. Considering that both hyalomin-A1 and -B1 could inhibit proinflammatory cytokine secretion by acting on JNK/SAPK signaling pathway and enable to scavenge free radical, Freund’s complete adjuvant-induced inflammation model was used to evaluate the potential anti-inflammatory ability of hyalomin-A1 and -B1. As illustrated in Fig. 5, they could inhibit hind paw inflammation in mouse in a dose dependent manner. Such anti-inflammatory functions were present significantly after 9 days administration. At the dose of 5 mg/kg body weight, inflammatory mice could recover to normal status after 21 days administration of hyalomin-A1 or -B1.

**DISCUSSION**

The investigation of interactions between blood-feeding arthropods and their hosts reveal the mechanism for the transmission of vector-borne pathogens. As pool feeders, ticks could be excellent models for the study of parasite-host relationships. Hard ticks, by virtue of their protracted feeding period (at least on week), represent an extreme example of interfering with the host immune responses. Their saliva or salivary gland secretions have the ability to suppress host immune responses and enhance the transmission of tick-borne pathogens. About 10 proteins from tick salivary glands have been identified to exert immuno-modulatory functions. One of them, Salp15 is a major immuno-modulatory protein in *Ixodes scapularis* saliva. Salp15 inhibits T cell receptor ligation-induced T cell signaling by binding to CD4. So far, no small peptides with immuno-modulatory ability have ever been characterized from ticks.

The current work identified two families of immuno-modulatory peptides (hyalomin-A and -B) from the salivary glands of the hard tick *H. asiaticum asiaticum*. They are small peptides with molecular weights of only 1231.16 and 3688.2. Hyalomin-A and -B are not found similar with any proteins already known. The cDNA cloning result further confirmed that these two peptides are encoded by a specific gene. Hyalomin-A and -B act as immunoregulators by inhibiting secretions of pro-inflammatory cytokines induced by LPS and increasing the secretion of immunosuppressant cytokine, IL-10. Interestingly, it was found that both hyalomin-A and -B could rapidly scavenge oxidants within several seconds. Mutant analysis indicated that the tyrosines in their sequences are responsible for the binding and next scavenging the oxidant. The rapid acting kinetics of free radical-scavenging of salivary peptides likely facilitates the escape of ticks or tick-borne pathogens from host oxidant stress. There are various evidences of cross-talk between inflammation and oxidants (42). The
antioxidant activities of hyalomin-A1/-B1 may contribute to their immunoregulatory and anti-inflammatory ability.

Many proofs have indicated that MAPK signaling pathway is involved in regulating the transcriptions of cytokine genes. Results from western blot analysis indicated that both hyalomin-A1 and -B1 significantly suppressed the LPS-induced activation of JNK subgroup of MAPK signaling pathway through blocking JNK’s phosphorylation, and subsequently led to the reduction of transcriptions of MCP-1, IFN-γ and TNF-α genes (Fig. 4). In addition, some literatures have reported that antioxidants can reduce oxidant-induced MAPK activation (42), thus the inhibitions of MAPK activation by hyalomin-A1/-B1 also might result from their antioxidant activates. It has been well established that components of the MAPK pathway act as mediators of phosphorylation of intracellular substrates such as protein kinases and transcription factors as well as regulators of cell growth and differentiation (43, 44). Given the inhibitory abilities of hyalomin-A1/-B1 on MAPK activation, we tested their effects on mouse splenocytes proliferation. A statistically significant reduction in the proliferation of cells could be observed for most of hyalomin-A1/-B1 concentrations tested (Fig. S2A). Cell viability has not been affected by hyalomin-A1/-B1, indicating that the immunoregulatory properties of hyalomin-A1/-B1 are not related with cytotoxicity.

In addition to the investigation of antioxidant and anti-inflammatory functions at molecular and cell levels, adjuvant-induced inflammatory models in mice were also exploited to evaluate the roles hyalomin-A1/-B1 playing in the acute inflammation. A concentration-dependent inhibition of inflammation was observed. This result further reveals the biological significance of these antioxidant peptides derived from tick salivary glands, replying on which the ticks could suppress host’s inflammatory responses and successfully get blood meal.

The current work identified two families of small peptides other than big proteins with antioxidant and anti-inflammatory activities from hard tick’s salivary glands. Besides, the possible mechanisms underlying their immunoregulatory functions were fully discussed.

**Supplemental material**

Fig. S1 shows the purification of immunosuppressant peptides from the tick salivary gland extracts; Fig. S2 shows effects of hyalomin-A1 and -B1 on cell proliferation and cell viability; Fig. S3 shows the MALDI data for the peptide analysis of hyalomin-A1 and -B1; Table S1 lists antioxidant activities of hyalomin-A1 and -B1.

**REFERENCES**


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**FOOTNOTES**

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FIGURE LEGENDS

Fig. 1. A: The cDNA sequence encoding hyalomin-A1 and -B1 from the hard tick *H. asiaticum asiaticum* salivary glands. The mature hyalomin-A and –B were boxed; B: the amino acid sequences of mature hyalomin-As and –Bs.

Fig. 2. Effects of hyalomin-A1 and -B1 on cytokine secretions induced by LPS. A: IFN-γ; B: IL-10; C: MCP-1; D: TNF-α. All values are means ± SD of three separate experiments. The values treated by hyalomin-A1 and –B1 are significant different from the values for controls (*P < 0.05 and **p < 0.01).

Fig. 3. Antioxidant functions of hyalomin-A1 and -B1. ABTS+ was scavenged by hyalomin-A1 and –B1 in a time-dependant manner. Water (A), BHT (B) and hyalomin-A1 (C) and –B1 (D) was added to standard ABTS+ solutions, respectively to a final concentration of 3 μM and the absorbance spectrum was read at 0 s, 2s, 30 s, 2 min, 5 min, 10 min, 20 min and finally endpoint scan was read immediately after the addition of sodium azide.

Fig. 4. The effects of hyalomin-A1 and -B1 on the LPS-induced phosphorylations of MAP kinase. Raw 264.7 macrophage cell were pretreated or not with the indicated concentrations of hyalomin-A1 and -B1 for 1 h before adding LPS (1 μg/ml) to the cells. After incubation for 15 min, total cellular proteins were prepared and Western blotted for p-IκB-α, p-p38, p-MKK4, p-JNK using specific antibodies. β-actin was used as internal control. For the Western blotting of p-C-Jun and p-ATF-2, cellular nuclear extracts were prepared, using PCNA as internal control. p: phosphorylated.

Fig. 5. Inhibition of adjuvant-induced paw inflammation by hyalomin-A1 (A) and -B1 (B). The number of animals per experiments is 12. All values are means ± SD of nine separate experiments. The values for paw thickness treated by hyalomin-A1 and –B1 are significant different from the values for controls (*P < 0.05 and **p < 0.01).
Figure 1

A

tatcaacgcagagtacgcgggggcacaccattggaggccaacctgctttgcacgtctcgg  60
gcggacaggaagatctcgaacgaaagaatgaactatctgtgcctagtagtgaccctcgtc 120
MNLYCLLVTVL

gtggctgggtgcaattttctggagagaaattttctgatgacaaatattgataccagtct 180
AVAGAISGEKFSDDNTTYQGS
actcaggeratttcaggtcaccaccaaggcgctccgacgcgtgacgacacccagctc 240

TTLRITTTPGRRRQTPRIT  51
gtccgccttaacccggacgcacgctgagaacgccagggattactcgcactacagtggaag 300

SPPYTRRRRLRRTTTDYSTTVE  71
aatttggactcactacccggccgcgttaaatagcaccggaaagggggagacacctgtgcg 360
NGNLTTPAAPANSEKGNGLYG

ccttcaccccgcagcagacgcagggattactgcgcctaaccctccgcgcgtggacgctc 420

LRRQTPRITGPPYT  111
acgccgggttactggactacagtggaaggggaaatggacactacccgggccgttaaatagc 480
TEGRNYVTVEKGNCTTPAANS
accggagggagccggccttacccggccgacgcacaaaccgccggacacccagctcggc 540

TEKGNRPYPGRRRTTPRITG  151
ccttcaccccgcagcagacgcagggattactgcgcctaaccctccgcgcgtggacgctc 600

PYPTRRRRTTDTWAAVEKEGLYT  171
acaccggccgaaacacgcacggaaaggggagacacgaacccggttcgacccagcagag 660

TPAAPANSEKESRPNATQRRE  191
acctcgatttcctttggccgcgtttaacccctggtggaccaaccaagggttacggagcaca 720
ISWTFGPLYTWRRTKGYGT  211
cctggaaacgcacaaatgcacaccccacgcagctag  753
LETNTSTS*  221

B
Hyalomin A1  QTPRTI GPPYT  11
Hyalomin B1  TLTTRTDYSTTVENGLTTPAAPANSEKGNGLYLQ  34
Hyalomin B2  TLTTRTDYYVTVEKCNTPAAPANSEKGRPYG  33
Hyalomin B3  TTDDYWAAVEKCYLTTPAAPANSEKESRPNATQ  32
Figure 2

(A) IFN-γ levels
(B) IL-10 levels
(C) MCF-1 levels
(D) TNF-α levels

Peptide concentration (μg/ml)
Figure 3

[Graphs showing absorption spectra for different conditions labeled A, B, C, and D. Each graph includes a wavelength range and absorbance values at specific wavelengths.]
### Figure 4

<table>
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<th>LPS (1 μg/ml)</th>
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<th>+</th>
<th>+</th>
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<th>+</th>
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<td>Hyalomin-A1 (μg/ml)</td>
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<td>2</td>
<td>4</td>
<td>8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hyalomin-B1 (μg/ml)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>5</td>
<td>8</td>
</tr>
</tbody>
</table>

- **IkB-α**
- **P-38**
- **Erk**
- **MKK4**
- **JNK**
- **β-actin**
- **C-JuN**
- **p-ATF-2**
- **PCNA**
Figure 5

(A) Right paw thickness (cm) over days after administration for Arthritis control, Hyalomin-A1 (1.25 mg/kg), Hyalomin-A1 (2.5 mg/kg), Hyalomin-A1 (5 mg/kg), and Normal control.

(B) Right paw thickness (cm) over days after administration for Arthritis control, Hyalomin-B1 (1.25 mg/kg), Hyalomin-B1 (2.5 mg/kg), Hyalomin-B1 (5 mg/kg), and Normal control.
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Jing Wu, Yipeng Wang, Han Liu, Hailong Yang, Dongying Ma, Jianxu Li, Dongsheng Li, Ren Lai and Haining Yu

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