

Original Article

High-mobility group protein N2 (HMGN2) inhibited the internalization of *Klebsiella pneumoniae* into cultured bladder epithelial cells

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Since bacterial invasion into host cells is an important step in the infection process, using the agents to interfere with bacterial internalization is an attractive approach to block the infection process. In this work, we describe a new, previously unrecognized role of the human cationic host defense peptide HMGN2 during *Klebsiella pneumoniae* infections. Our results revealed that the internalization of *K. pneumoniae* strain 03183 into cultured bladder epithelial cells (T24) was significantly reduced at HMGN2 concentrations that were unable to produce any bacteriostatic or bactericidal effect. Using microarrays and follow-up studies, we demonstrated that HMGN2 affected the internalization of *K. pneumoniae* strain 03183 by inhibiting the attachment of bacteria, and then decreasing bacteria-induced ERK1/2 activation and actin polymerization, which might contribute to bacterial internalization into T24 cells. This disruption of bacterial internalization implied that HMGN2 could provide protection against *K. pneumoniae* infections.

Keywords bladder epithelial cells; internalization; HMGN2; *Klebsiella pneumoniae*

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Introduction

Klebsiella pneumoniae is recognized as an important opportunistic pathogen that frequently causes urinary tract infections (UTI), septicemia, or pneumonia, particularly in immunocompromized individuals [1]. *K. pneumoniae* is responsible for up to 10% of all nosocomial bacterial infections [2]. Furthermore, *K. pneumoniae* isolates resistant to antibiotics mediated by extended spectrum β -lactamases have now spread worldwide [3,4]. Due to the emergence of multidrug resistance among *Klebsiella* strains, the search for new approaches for the prevention or treatment of *Klebsiella* infections is now under intensive investigation [5].

Host cell infection begins with the internalization of the bacteria, which includes a complex series of interactions between the bacteria and the cell [6]. Some researchers have suggested that *K. pneumoniae* internalization represents a virulence mechanism, because it enables the bacteria to avoid host defenses and antimicrobial drugs and therefore might represent a reservoir of the pathogen [7]. Thus, the inhibition of internalization may serve as an additional mechanism to prevent bacterial survival in the host. Low concentration of secretory immunoglobulin A, lactoferrin and free secretory component inhibit adhesion and, consequently, invasion of *Shigella*, thereafter prevent shigellosis in infants [8]. *Phyllanthus urinaria* chloroform inhibits the *Helicobacter pylori* adhesion to and invasion of human gastric adenocarcinoma epithelial (AGS) cells *in vitro* [9].

HMGN2 has been described as an abundant family of non-histone proteins in the cell nucleus of vertebrate and invertebrate organisms [10]. Series of experiments have shown that HMGN2 is preferentially associated with gene transcription and organogenesis [11]. Recently, our group has isolated an antimicrobial polypeptide from human lympholine-activated killer (LAK) cells and cervical mucus, which is characterized to be the HMGN2 [12,13]. Until now, the biological role of this protein has not been fully defined. Our previous work has illustrated that the transmembrane α -helical structure located in the 17–47 residues, which has been found to be the DNA-binding domain of HMGN2, is essential for its antibacterial activity against *Escherichia coli* ML-35p, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 10231, and, to some extent, against human hepatitis B virus [13,14].

In the present study, we demonstrated that HMGN2 at non-bacteriostatic or non-bactericidal concentration obviously prevented *K. pneumoniae* strain 03183 to internalize into T24 cells. The changes of bacterial adhesion and host cellular responses that were responsible for bacterial uptake were investigated after *K. pneumoniae* strain 03183 pretreated with HMGN2.

Materials and Methods

Strains and cell culture

K. pneumoniae strain 03183 was isolated from the sample of urinary tract infection patient at the Department of Medical West China Hospital of Sichuan University (Chengdu, China). Hemagglutination assays confirmed that *K. pneumoniae* strain 03183 was able to produce type 1 fimbriae. Bacteria were grown overnight at 37°C in Luria Broth (LB) medium, and then 100 µl of this culture were grown in LB medium until mid-log-phase, washed, and resuspended with PBS. Human bladder epithelial cell line T24 was cultivated in RPMI-1640 medium (Gibco, Carlsbad, USA) with 10% bovine serum (Gibco) in 5% CO₂ at 37°C.

Heat-killed bacteria

Bacteria were grown and prepared as above, then incubated for 30 min at 65°C, cooled to room temperature, and used for experiments.

Recombination of human HMGN2

Recombinant human HMGN2 was prepared as previously described [13,14]. Briefly, the transformed *E. coli* BL21 (λDE3) carrying pET-32a (+)-HMGN2 was cultured in LB medium in the presence of 0.1 mM IPTG to induce protein expression. The cell lysates were obtained by freezing/thawing in the presence of lysozyme. The fusion proteins were purified using HisTrap Chelating HP columns (Pharmacia, Piscataway, USA), then cleaved by thrombin digestion. The chimeric protein HMGN2 was obtained by reverse-phase high-performance liquid chromatography on a 4.6 × 250-mm Vydac C18 column. Protein concentration was determined by bicinchoninic acid BCA Protein Assay Kit (Pierce, Rockford, USA), and the purity was confirmed by Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot with a primary anti-His6 monoclonal antibody (Sigma, St. Louis, USA). Unless otherwise noted, all experiments were performed with HMGN2 at a concentration of 128 µg/ml, which is a non-bacteriostatic and non-bactericidal concentration.

Antibacterial activity

Bacteria (1×10^9 cfu/ml) were incubated at 37°C for 2 h PBS supplemented with different concentrations of HMGN2. Then the reaction mixtures and appropriate controls were plated on LB agar to determine the colony-forming unit as described previously [15].

Bacterial internalization assay

Bacterial internalization assay was performed as described previously [5,15,16]. *K. pneumoniae* strain 03183 was

pretreated with HMGN2, PBS, and BSA, respectively, for 2 h at 37°C before infection. T24 cells were grown in 24-well cell culture clusters to confluent monolayers (2×10^5 cells per well) in RPMI-1640 medium with 10% bovine serum. T24 cells were infected at multiplicity of infection (MOI) of 100 bacteria per cell for 2 h at 37°C [17]. Then cells were washed thrice with PBS, and gentamicin (Sigma) diluted in fresh PBS was added to each well at the final concentration of 100 µg/ml to kill extracellular bacteria. After incubation for 2 h at 37°C, cells were washed with PBS, lysed by addition of cold 0.1% Triton X-100, and plated on LB agar to determine the viable intracellular bacteria. Internalization ability was expressed as the number of internalized bacteria per T24 cell.

Microarray analysis

K. pneumoniae strain 03183 was grown in LB medium as described above in the absence or presence of HMGN2. After 2 h incubation, bacteria were washed thrice with PBS. The total RNA was isolated from bacteria with Trizol reagent (Invitrogen, Carlsbad, USA) for mRNA Microarray analysis. mRNA hybridization was performed by ShanghaiBio Corporation (Shanghai, China) using Agilent Genome Oligo Microarray (total 4820 oligo probes). Hybridized arrays were scanned at 5 µm resolution on an Agilent DNA Microarray Scanner. Data extraction from images was done by using Agilent Feature Extraction software. *K. pneumoniae* strain MGH78578 was used to determine the genome sequence of *K. pneumoniae* strain 03183, and *E. coli* str. K-12 DNA was used to analyze the genome of *K. pneumoniae* [18,19].

Bacterial adhesion assay

T24 cells were infected at MOI of 100:1 for 1 h at 37°C in 5% CO₂. After infection, cell monolayers were washed carefully thrice with PBS to remove unattached bacteria, lysed by addition of cold 0.1% Triton X-100, and plated on LB agar to determine the number of bound bacteria. Adherence ability was expressed as the number of associated bacteria per T24 cell [20].

Inhibition assay

Cytochalasin B (Sigma), a microfilament depolymerization agent, and PD98059 and U0126 (Sigma) the MAPK kinase (MEK1/2) inhibitors were used for inhibition assay. All inhibitors were dissolved in dimethyl sulphoxide (DMSO). The concentration of each inhibitor employed was chosen for inhibitory effect without affecting the epithelial cell. The inhibition on eukaryotic cell was studied by adding individual inhibitors to the monolayer 1 h prior to the addition of bacteria. Inhibitors were maintained throughout the 2-h internalization period. The internalization ability was calculated as described above.

Western blot

T24 cells were infected as described above. After 1 h of infection, cells were harvested using the Cytoplasmic Extraction kit (Pierce) according to the manufacturer's instructions. The protein concentration was determined with a BCA protein assay kit (Pierce). Total protein (40 µg) from each sample was subjected to 10% SDS-PAGE analysis. Proteins were transferred to the polyvinylidene difluoride membranes using a wet blotting apparatus (Bio-Rad). The primary and secondary antibodies were diluted with TBST containing 5% skimmed milk. After 1 h of incubation with the appropriate primary antibody (polyclonal rabbit anti-phospho-ERK1/2, mouse anti-ERK1/2; 1:1000; Sigma), membranes were washed four times 15 min each time, in TBST and the appropriate secondary peroxidaseconjugated antibody (goat anti-rabbit, goat anti-mouse; 1:5000; Sigma) was added for another hour. The membranes were treated using ECL reagents (GE Healthcare, Pennsylvania, USA). Western blots were analyzed by densitometry using Image Lab software (Bio-Rad). Results presented are the average of three independent experiments [20].

Immunofluorescence microscopy

T24 cells were infected by *K. pneumoniae* pretreated with HMGN2 or PBS (as control). Coverslips were washed thrice in PBS and fixed with 4% paraformaldehyde for 15 min. For immunostaining, cells were permeabilized for 10 min in PBS containing 0.5% Triton X-100, washed three times in PBS. The coverslips were blocked for 1 h with PBS containing 0.5% BSA and incubated for 30 min with rhodamine-conjugated phalloidin (for actin, Sigma) and 4',6'-diamidino-2-phenylindole (DAPI, for cell nucleus, Sigma). Coverslips were visualized by a Zeiss immunofluorescence microscope (×20 objective giving a total magnification of ×200; (Carl Zeiss, Jena, Germany).

Flow cytometry

A confluent monolayer of T24 cells was infected with *K. pneumoniae* pretreated with HMGN2 or PBS (as control) at MOI of 100:1 for 2 h at 37°C in 5% CO₂. T24 cells were then washed vigorously, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with rhodamine-conjugated phalloidin for 30 min. Flow cytometry analysis was performed with an EpicsXL Coulter flow cytometer (Beckman, Fullerton, USA) and repeated thrice.

Statistical analysis

Data are expressed as the means ± SD. Statistical analyses were performed using the Student's *t*-test. *P* < 0.05 was considered statistically significant.

Results

HMGN2 inhibits the internalization of *K. pneumoniae* strain 03183 into T24 cells

The antibacterial activity of HMGN2 on *K. pneumoniae* strain 03183 was investigated. *K. pneumoniae* strain 03183 was incubated with different concentrations of HMGN2. Up to 512 µg/ml HMGN2 did not affect bacterial viability (Table 1). Growth of *K. pneumoniae* strain 03183 was measured with different concentrations of HMGN2. No inhibition on the bacterial growth was observed (data not shown). Then we tested the effect of HMGN2 on the internalization of *K. pneumoniae* strain 03183. The results showed that the number of viable bacteria into T24 cells was significantly decreased in 128 µg/ml HMGN2 group compared with that in the control (0.16 ± 0.04 vs. 0.31 ± 0.03 , *P* < 0.05) (Fig. 1). With the increase of HMGN2 concentration, the inhibition on internalization became

Table 1 Antibacterial activity of HMGN2 toward *K. pneumoniae* strain 03183

HMGN2 concentration (µg/ml)	Viability of bacteria (cfu/ml) ^a
0	1.0×10^9
8	1.0×10^9
32	1.1×10^9
128	1.1×10^9
256	1.1×10^9
512	1.2×10^9

^a*K. pneumoniae* strain 03183 was cultivated in LB for 16 h at 37°C. The bacteria was pelleted by centrifugation and washed with PBS. The bacteria was resuspended in PBS at 1.0×10^9 cfu/ml, and HMGN2 was added at different concentrations. The viable cell count was determined after incubating at 37°C for 24 h.

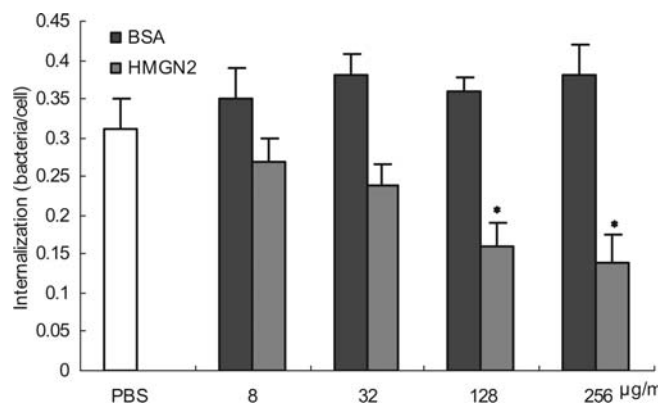


Figure 1 Effect of HMGN2 on *K. pneumoniae* strain 03183 internalization. *K. pneumoniae* strain 03183 were pretreated with different concentrations of HMGN2, BSA, and PBS (as control), then infected T24 cells at an MOI of 100:1 for 2 h. The numbers of intracellular bacteria were determined by the viable plate count method. This study was repeated at least thrice. **P* < 0.05 vs. control bacteria.

more significant (0.14 ± 0.04 vs. 0.31 ± 0.03 , $P < 0.05$). It was found that BSA had no inhibitory effect on *K. pneumoniae* strain 03183. These results showed that HMGN2 prevented *K. pneumoniae* strain 03183 to internalize into T24 cells.

Microarray analysis

To illustrate the mechanism of internalization inhibition, we examined the effect of HMGN2 on the gene expression profile of *K. pneumoniae* strain 03183. Microarray analysis was performed to analyze the global gene expression of bacteria in the presence or absence of HMGN2 [21]. Three independent experiments were performed. Forty-one genes displayed statistically significant expression levels ($P < 0.05$). Among these genes, 22 genes were up-regulated and 19 down-regulated. Among these 22 genes with increased expression levels, $>50\%$ were ribosomal protein genes, which were involved in tRNA metabolism or related to nucleic acid metabolism. Among other 19 genes, 40% were related to bacterial adhesion and 20% corresponded to bacterial virulence. In addition, a number of genes of which functions are unknown yet showed significantly decreased expression levels. Down-regulated genes related to bacterial adhesion are shown in **Table 2**.

Type 1 fimbriae are related to bacterial adhesion. Interestingly, *fimA*, *fimB*, and *fimH* genes that are involved in the type 1 fimbria gene cluster of *K. pneumoniae* and encode the major fimbrial subunit protein FimA, the recombinase FimB, and the adhesin protein FimH, respectively, were down-regulated. Meanwhile, we also observed the down-regulation of several Cpx two-component system-controlled genes in the presence of HMGN2.

HMGN2 inhibits the adherence of *K. pneumoniae* strain 03183 to T24 cells

Bacterial adherence to host cells or surfaces is often an essential first stage in disease, and the adherence to host

cells may result in internalization [16]. In this study, we observed that down-regulated genes were associated with adhesion, and investigated the attachment behavior of bacteria in the presence of HMGN2. Attachment assays were carried out at different HMGN2 concentrations. HMGN2 obviously decreased the attachment of *K. pneumoniae* to T24 cells at a concentration of 128 $\mu\text{g/ml}$ (4.20 ± 0.34 vs. 7.72 ± 0.44 , $P < 0.05$), and with the increase of the concentration, the inhibition effect became more significant (**Fig. 2**).

Internalization of *K. pneumoniae* strain 03183 into T24 cells depended on ERK1/2.

To test whether MEK and ERK signal transduction involved in the internalization of *K. pneumoniae* strain 03183 into T24 cells, we detected the levels of p-ERK1/2 in T24 cells when exposed to *K. pneumoniae* strain 03183 at an MOI of 100:1 for different periods of time. Immunoblots revealed the phosphorylation of ERK1/2 occurred as early as 10 min after infection and reached to the peak 30–60 min later. ERK1/2 phosphorylation in T24 cells continued for at least 2 h post-infection [**Fig. 3(A)**]. Similar to live *K. pneumoniae* strain 03183, heat-killed bacteria still induced activation of the ERK1/2 [**Fig. 3(B)**]. However, there was five-fold decrease in ERK1/2 phosphorylation at the same time-point when T24 cells were infected at an MOI of 25:1 [**Fig. 3(C)**].

When T24 cells were pretreated with PD98059 (30 μM) or U0126 (10 μM) for 1 h prior to bacterial infection, the numbers of intracellular bacteria reduced as much as 67 and 60% compared with control [**Fig. 4(A)**]; and immunoblot results showed that PD98059 and U0126 strikingly inhibited the activation of ERK1/2 [**Fig. 4(B)**]. These results illustrated that the internalization of *K. pneumoniae* strain 03183 into T24 cells depended on ERK1/2.

To examine whether attachment or invasion of *K. pneumoniae* strain 03183 stimulated ERK1/2 phosphorylation, normal T24 cells were treated with cytochalasin B

Table 2 Selected *K. pneumoniae* 03183 genes that were down-regulated in the presence of HMGN2

b NO	Designation	Regulation	Change in regulation (fold)	Protein
Type 1 fimbriae				
b4314	<i>fimA</i>	down	2.034	Major type 1 subunit fimbrin
b4312	<i>fimB</i>	down	1.501	Recombinase involved in phase variation; regulator for <i>fimA</i>
b4317	<i>fimD</i>	down	1.712	Putative fimbriae; usher
b4320	<i>fimH</i>	Down	1.521	Minor component of type 1 fimbriae
Two-component system				
b3911	<i>cpxA</i>	Down	1.691	Two-component sensor protein
b3912	<i>cpxR</i>	Down	1.787	Hypothetical protein
b0161	<i>htrA</i>	Down	1.699	Periplasmic serine protease Do, heat shock protein
b3806	<i>dsbA</i>	Down	1.726	Periplasmic protein disulfide isomerase I

b NO, a unique identifier for *Escherichia coli* genes.

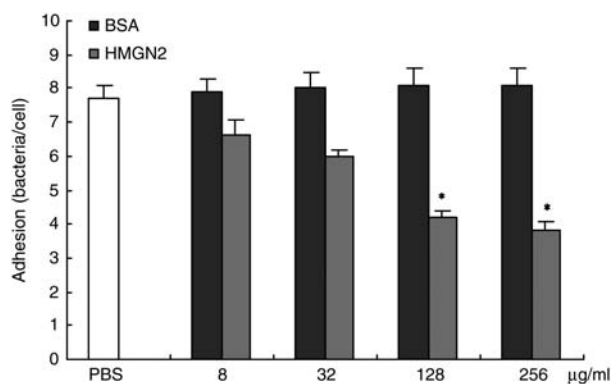


Figure 2 Effect of HMGN2 on *K. pneumoniae* strain 03183 adhesion *K. pneumoniae* strain 03183 were pretreated with different concentrations of HMGN2, BSA, and PBS (as control), then infected T24 cells at an MOI of 100:1 for 1 h. The numbers of adherent bacteria were determined by the viable plate count method. This study was repeated at least thrice. * $P < 0.05$ vs. control bacteria.

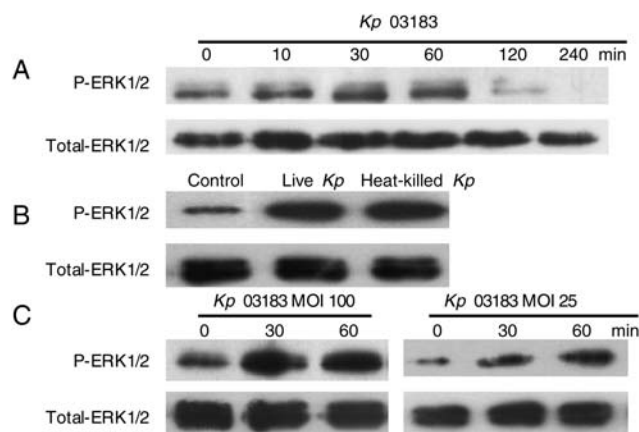


Figure 3 Activation of ERK1/2 in T24 cells infected by *K. pneumoniae* strain 03183 (A) T24 cells were either uninfected (0 min) or infected with *K. pneumoniae* strain 03183 at an MOI of 100:1 for various times (10, 30, 60, 120, 240 min). (B) T24 cells were either uninfected (0 min) or infected with live and heat-killed *K. pneumoniae* strain 03183 at an MOI of 100:1 for 60 min. (C) T24 cells were either left uninfected (0 min) or infected with *K. pneumoniae* strain 03183 for various times (30, 60 min) at an MOI of 100:1 and 25:1.

(10 µg/ml) prior to bacterial infection. Results showed that cytochalasin B apparently inhibited the invasion of *K. pneumoniae* strain 03183 >90% compared with control [Fig. 4(A)]. Meanwhile, the ERK1/2 phosphorylation levels were determined. The increase in ERK1/2 phosphorylation observed in response to bacteria infection was unaffected by treatment with cytochalasin B [Fig. 4(B)]. Thus, we concluded that ERK1/2 activation occurs prior to bacterial invasion.

HMGN2 pretreatment decreases

K. pneumoniae-induced ERK1/2 activation in T24 cells

As shown in Fig. 5, ERK1/2 phosphorylation increased significantly in cells exposed to *K. pneumoniae* strain

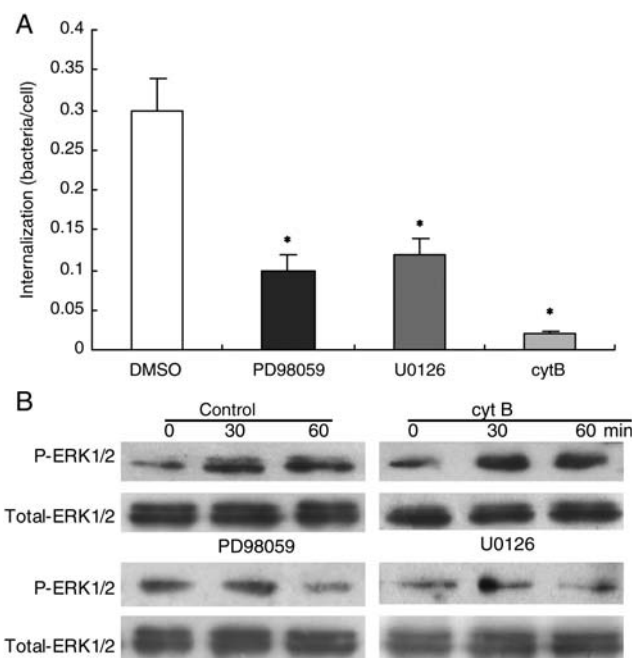


Figure 4 Effect of inhibitors on the internalization of *K. pneumoniae* strain 03183 (A) and ERK1/2 activation (B) (A) PD98059 (30 µM), U0126 (10 µM), cytochalasin B (10 µg/ml), and DMSO (as control) pretreated with T24 cells for 1 h, then *K. pneumoniae* strain 03183 infected T24 cells for 2 h. The numbers of intracellular bacteria were determined by the viable plate count method. This study was repeated at least thrice. * $P < 0.05$ vs. control cells. (B) T24 cells were pretreated with DMSO (control), PD98059 (30 µM), U0126 (10 µM), and cytochalasin B (10 µg/ml) for 60 min. T24 cells were then either left uninfected (0 min) or infected with *K. pneumoniae* strain 03183 for various times (30, 60 min).

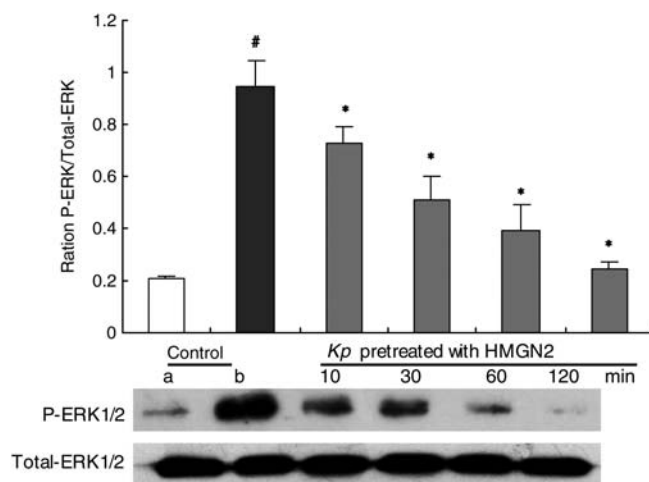


Figure 5 HMGN2 pretreatment decreased *K. pneumoniae*-induced ERK1/2 activation in T24 cells (a) Uninfected T24 cells. (b) T24 cells were infected by untreated *K. pneumoniae* strain 03183 for 60 min. *K. pneumoniae* strain 03183 were pretreated with HMGN2 at different periods of time from 10 min to 120 min and then infected T24 for 60 min. Quantification of densitometry was conducted using Image Lab software and results presented here represented the mean \pm SD of three independent experiments. * $P < 0.05$ vs. uninfected cells, * $P < 0.05$ vs. cells exposed to untreated bacteria.

03183 compared with uninfected cells. *K. pneumoniae* strain 03183 pretreated with HMGN2 also could induce ERK1/2 phosphorylation in T24 cells, but the levels of p-ERK1/2 were lower than that in untreated bacteria. Meanwhile, the low levels of ERK1/2 phosphorylation were observed as early as 10 min after pretreatment.

HMGN2 pretreatment decreases

K. pneumoniae-induced actin polymerization in T24 cells

To examine the effects of HMGN2 pretreatment on the cytoskeletal F-actin network, we stained T24 cell with rhodamine-conjugated phalloidin. *K. pneumoniae* strain 03183 induced actin polymerization. The brightest red fluorescence could be observed at the periphery of cells and actin stress fiber was induced in most of the infected cells. *K. pneumoniae* strain 03183 pretreated with HMGN2 also induced actin polymerization, but the degree of polymerization decreased compared with that induced by untreated bacteria (Fig. 6). The contents of F-actin were expressed as mean fluorescent intensity. As illustrated in Fig. 6(E), pretreatment with high concentration of HMGN2 resulted in a significant decrease in F-actin content compared with that induced by untreated bacteria.

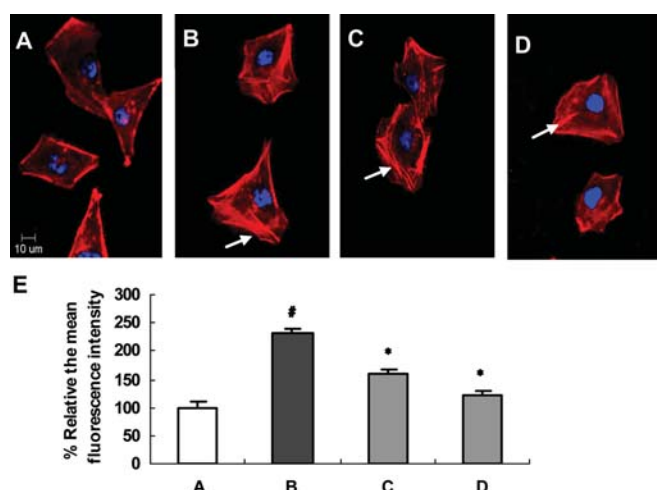


Figure 6 HMGN2 pretreatment decreased *K. pneumoniae*-induced actin polymerization in T24 cells (A) Uninfected T24 cells. (B) T24 cells were infected by unpretreated *K. pneumoniae* strain 03183. (C, D) T24 cells were infected by *K. pneumoniae* strain 03183 pretreated with 128 µg/ml and 256 µg/ml HMGN2, respectively. F-actin was labeled with rhodamine-conjugated phalloidin (red) and cells nucleus were labeled with DAPI (blue). White arrows show actin stress fibers. (E) Mean fluorescence intensity was shown in each group. Relative the mean fluorescence intensity in uninfected T24 was defined as 100%. Each column represents the average of at least three results in duplicate. [#]*P* < 0.05 vs. uninfected cells, ^{*}*P* < 0.05 vs. cells exposed to untreated *K. pneumoniae* strain 03183.

Discussion

The current results confirmed that HMGN2 significantly inhibited the internalization of *K. pneumoniae* strain 03183 into T24 cells *in vitro*, which occurred at non-bacteriostatic and non-bactericidal concentration of HMGN2. The interaction of HMGN2 with *K. pneumoniae* strain 03183 is not fully understood yet; however, other antimicrobial peptides are known to have various targets against bacteria, including bacterial membranes, DNA, RNA, and cellular proteins, among others [21,22]. However, we demonstrated that the inhibition of internalization could reflect HMGN2-mediated impaired adherence of bacteria to T24 cells, and then modulated host cellular responses leading to the disruption of internalization process.

Through microarray analysis, several genes related to bacterial adherence were down-regulated in the presence of HMGN2. Most clinical *K. pneumoniae* isolates are able to produce type 1 fimbriae and type 3 fimbriae [23]. Hemagglutination assays proved that *K. pneumoniae* strain 03183 can express type 1 fimbriae. Type 1 fimbriae and the adhesive subunit FimH play important roles in UTI caused by *K. pneumoniae* [3,23]. However, several genes associated with the function of type 1 fimbriae were found to be down-regulated in the presence of HMGN2. On the other hand, we also observed that HMGN2 affected the Cpx two-component regulatory system of *K. pneumoniae* strain 03183. Studies have implicated the Cpx envelope stress response in the regulation of early steps in infection, including adherence and possibly invasion, in several pathogens [24]. To test the importance of these gene expression changes, the attachment of *K. pneumoniae* strain 03183 was analyzed in the presence of different concentrations of HMGN2. The present results revealed that HMGN2 obviously inhibited the adherence of *K. pneumoniae* strain 03183 to T24 cells. Meanwhile, with the increasing concentration of HMGN2, the adhesion inhibition became more significant (Fig. 2).

Adherence usually triggers or activates signals in host cells that directly or indirectly mediate and facilitate bacterial entry. Thus, bacterial invasion is an active event that relies on underlying normal host cell functions [25]. We have learned that the adhesion of *K. pneumoniae* strain 03183 was inhibited by HMGN2. Subsequently, we evaluated the changes of host cellular responses that were responsible for bacterial uptake in response to the reduced adherence.

To gain entry into host cells, most invasive bacterial pathogens exploit pre-existing host signal transduction cascades [26]. For example, clustering of fibronectin-bound $\beta 1$ integrin receptors by *streptococcal* SfbI triggers the recruitment of the Rho GTPases Cdc42 and Rac, as well as

recruitment/phosphorylation of focal adhesion kinase, producing actin rearrangements that eventually lead to bacterial internalization [27]. Src and PI 3-kinase has been implicated in the regulation of events involved in the alteration of the actin cytoskeleton [28,29] and the roles in bacterial invasion of host cells have been reported [30,31]. Recent studies have identified MEK and ERK signaling proteins play very important role in the invasion of bacteria, such as *P. aeruginosa*, *Staphylococcus aureus* and *Listeria monocytogenes* [27,32,33]. Our reports proved that *K. pneumoniae* induced ERK1/2 activation in T24 cells. Moreover, ERK1/2 phosphorylation was observed during both heat-killed and live bacteria infection [(Fig. 3(B)). There are reports showing that *K. pneumoniae* expresses molecules, such as lipopolysaccharide (LPS), which trigger the activation of Toll-like receptors (TLRs) [34,35]. Wu *et al.* [36] suggested that *K. pneumoniae* infections induce signal transduction through TLR2 and TLR4 and activate downstream MAP kinase pathways. PD98059 and U0126 significantly blocked bacterial invasion [Fig. 4(A)]. These suggested that the internalization of *K. pneumoniae* strain 03183 into T24 cells was dependent on MEK–ERK signal proteins.

To examine whether bacterial attachment was responsible for the activation of ERK1/2, T24 cells were pretreated with cytochalasin B. ERK1/2 phosphorylation was still induced when T24 cells were treated with cytochalasin B, indicating that ERK1/2 activation occurred prior to bacterial invasion. Meanwhile, ERK1/2 phosphorylation was dependent on *K. pneumoniae* MOI. When T24 cells were infected by bacteria pretreated with HMGN2, the levels of ERK1/2 phosphorylation obviously reduced compared with that induced by unpretreated bacteria. HMGN2 pretreatment decreased *K. pneumoniae*-induced ERK1/2 activation in T24 cells. These results indicated that the inhibition of *K. pneumoniae* strain 03183 adherence in the presence of HMGN2 induced the reduction of ERK1/2 activation.

Activated ERK1/2 probably effects cytoskeletal rearrangement by inducing actin reorganization [6,37]. Chu *et al.* [38] reported that the MEK-1 inhibitor PD98059 decreases ERK1/2 phosphorylation and blocked actin reorganization. They suggested that ERK1/2 signaling pathways might be activated and be necessary for integrin-mediated actin reorganization in embryonic avian corneal epithelium. Our investigation demonstrated that *K. pneumoniae* strain 03183 triggered an uptake mechanism into T24 cells, which depended on actin microfilaments. As shown in Fig. 5, *K. pneumoniae* strain 03183 pretreated with HMGN2 inhibited ERK1/2 phosphorylation compared with unpretreated bacteria. IFM and FMI showed that HMGN2 pretreatment decreased *K. pneumoniae*-induced actin polymerization (Fig. 6). Taken together, the decreased actin polymerization might be due to the reduced ERK1/2 activation, leading to prevent *K. pneumoniae* 03183 to internalize.

In conclusion, HMGN2 inhibited the internalization of *K. pneumoniae* strain 03183 into T24 cells through inhibiting bacterial adherence, subsequently decreasing host cells ERK1/2 activation and actin polymerization *in vitro*, which indicated that HMGN2 might be an important defense factor and might provide protection for the mucous membranes that are the initial target in most infections. Our further study will focus on the interaction between HMGN2 and *K. pneumoniae* during infection.

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