Biochemical and Biophysical Research Communications xxx (2017) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Effects of the RNA-binding protein, KSRP, on innate immune response against *Helicobacter pylori* infection in mice

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ARTICLE INFO

Article history:
Received 13 November 2017
Accepted 4 December 2017
Available online xxx

Keywords:
Helicobacter pylori
KSRP
Immune response
Infection

ABSTRACT

Helicobacter pylori (*H. pylori*) contributes to various gastric diseases such as chronic gastritis, gastric ulcer, and gastric carcinoma. Host innate immune response against the pathogen plays a significant role in elimination of pathogen infection. Importantly, pathogen elimination is closely related to numerous inflammatory-related genes that participate in complex biological response of cells to harmful stimuli. Here we studied effects of the KH-type splicing regulatory protein (KSRP), a RNA-binding protein, on innate immune response against *H. pylori* infection. We found that *H. pylori* infection downregulated KSRP expression directly, and that KSRP overexpression repressed upregulation of *CXCL-2* expression induced by *H. pylori* and facilitated *H. pylori* proliferation *in vitro*. Similarly, KSRP overexpression in *H. pylori* mice also facilitated *H. pylori* proliferation and colonization, and induced more severe gastric mucosal damage. Intriguingly, *CXCL-2* and *HMOX-1* were upregulated in *H. pylori* infected mice after KSRP overexpression. This difference in expression of these genes implicated that KSRP was closely associated with and directly participated in the innate immune response against *H. pylori*. These results were beneficial for understanding the *in vivo* function of KSRP on innate immune response against pathogen infection.

1. Introduction

Helicobacter pylori (*H. pylori*), a spiral gram-negative bacterium, plays an etiologic role in the development of various diseases such as chronic gastritis, gastric ulcer and duodenal ulcer [1]. More than half of the world's population is infected with *H. pylori*, which can lead to the development of gastritis and gastric carcinoma [2]. This organism colonizes the stomach [3–5] and triggers immune response through activation of multiple signaling pathways such as NF-κB and TLRs pathways [6]. It has been reported that *H. pylori* evaded the immune recognition mediated by TLR 5, a receptor specifically recognizing the bacterial flagellum [7]. However, the complicated mechanism causing severe gastric diseases by *H. pylori* remains unclear. Although considerable progress concerning therapy of *H. pylori* infection has been made, the detailed pathogenesis of *H. pylori* as still not well understood.

KSRP (also called FBP2), a KH domain RNA binding protein, was originally considered as a component of a protein complex that implicated in neuronal-specific splicing enhancer [8]. Subsequently, multiple functions of KSRP including AU-rich element associated mRNA decay, and as a component of the miRNA precursor processing complexes that participate in biogenesis of some microRNAs were identified [9]. Researchers revealed significant function of KSRP on controlling stability of select myogenic transcripts and on convergence of the PI3K and p38 MAPK pathways [10]. Recently, posttranscriptional control of several interferon genes by KSRP was also reported recently [11]. But the effects of KSRP on innate immune response against *H. pylori* infection do not know. Whether KSRP mediated regulation of innate immune response, is an epithelial anti-microbial defense process in general remains unclear.

Our previous study demonstrated that KSRP was engaged in the

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https://doi.org/10.1016/j.bbrc.2017.12.016
0006-291X/

innate immune response to lipopolysaccharide (LPS) stimulation. Here we questioned whether KSRP played a critical role in innate immune response against *H. pylori* infection both *in vitro* and *in vivo*. We discovered the important function of KSRP on expression regulation of some inflammatory factors mediated by *H. pylori* infection, suggesting that KSRP played a critical role in innate immune response against *H. pylori* infection.

2. Materials and methods

Cell and *H. pylori* culture. Human gastric epithelial cell line (GES-1) was routinely maintained in DMEM (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, AUS) at 37 °C incubator with 5% CO_2. The *H. pylori* strain (we called *H. pylori* SH1 strain), a clinically isolated strain, was preserved and identified by our laboratory, and cultured routinely on brain heart infusion-columbia agar media under microaerobic atmospheres condition (10% CO_2, 5%O_2, 85% N_2). Agar media supplemented with 7% sheep blood, 10 mg/mL vancomycin, 10 mg/mL amphotericin, 2500 U/L polymyxin B sulfate salt, 5 mg/mL trimethoprim, 10 mg/mL nalidixic acid (all obtained from Sigma, USA).

The phylogenetic relationships between the 16S rDNA sequences of *H. pylori* SH1 and homologous sequences of other *H. pylori* strains were analyzed by the neighbor-joining method implemented in MEGA 5 with default settings. The level of support for each node was evaluated by 1000 bootstrap replications.

Plasmid construction and transfection. pcDNA3.0 and pcDNA3.0-KSRP plasmid were preserved by our laboratory. Sequence identification of recombinant plasmid was verified by TsingKe biological technology company (Beijing China). Lipofectamine 2000 was used for plasmid transfection according to the manufacturer's instructions.

For *in vivo* transfection, cationic liposome was prepared as multilamellar vesicles as described previously [12]. Plasmid/lipoplexes were prepared immediately before injection by gently mixing cationic lipids with plasmid DNA at a ratio of 5:1 (liposome: DNA) to a final concentration of 25 μg plasmid DNA per ml in a sterile solution of 5% glucose. Tail intravenous injection was employed to *in vivo* transfection, and the mice only injected with 5% glucose were served as control.

***H. pylori* co-incubation.** *H. pylori* was harvested after cultured 3–5 d under microaerobic atmospheres condition. Cell culture medium was replaced by serum-free DMEM (Hyclone, USA) on the day of co-incubation. *H. pylori* suspension was counted and added in 6-well plates at 100:1 bacterium/cells ratio, and then sequentially incubated 12 h or 24 h at 37 °C with 5% CO_2.

qRT-PCR. Total RNAs of cells or tissues were extracted by Trizol (Invitrogen, USA) according to the manufacturer's instructions and quantified by agarose gel electrophoresis. 1 μg total RNAs of each sample were reverse-transcribed using PrimeScript™ RT reagent Kit (Takara, Japan) for qRT-PCR using SYBR Green master mix (Takara, Japan). PCR was performed by Bio-Rad iQ5 system using primer pairs listed in Table S1 and Table S2. GAPDH and β-actin were used as internal control for GES-1 cell samples and mice tissue samples respectively.

Western blot. Total protein was extracted by culture cell protein extraction reagents (Boster Biological Technology, China) containing 1% (v/v) phenylmethanesulfonyl fluoride (PMSF; Boster, China), after which protein concentrations were measured using BCA protein assay kit (Nanjing jiancheng bioengineering institute, China). 50 μg boiled total protein samples from each group were separated by 4–10% sodium dodecylsulfate polyacrylamide gel electrophoresis, and then transferred onto polyvinylidene fluoride membranes. After blocked by 5% (w/v) skimmed milk for 2 h at room temperature, the membranes were incubated with anti-KSRP (Abcam, UK) and anti-β-actin (GeneTex, USA) respectively at 4 °C overnight. Membranes were washed three times by TBST buffer and incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit IgG for 1 h subsequently. Finally, membranes were detected using ECL chemiluminescent labeling reagents (Boster, China) according to the manufacturer's protocols.

Determination of *H. pylori* number. After 12 h co-incubation with *H. pylori*, cell culture medium was collected and then diluted. 100 μL of each diluted liquid was coated uniformly on brain heart infusion-columbia agar. *H. pylori* number was counted after incubating 3–5 d in a 37 °C incubator under microaerobic atmospheres condition.

***H. pylori* infection in BALB/c mice.** All age-matched BALB/c mice weighing 18–22 g (24 male and 24 female, supplied by laboratory animal center of Sichuan University) were caged separately at 25–28 °C, 50%–70% relative humidity. Mice were inoculated with *H. pylori* suspension and given stranded diet throughout the experiment as previously described [13]. Briefly, total 48 BALB/c mice divided (equally among female and male) into 8 groups. Experimental groups were orally inoculated with 10^8 CFU *H. pylori*-SH1 strain in 0.3 mL of PBS given three times every other day. Control mice received sterile PBS only. At 1 month after challenge, six infected mice were sacrificed and the gastric tissues were assessed by rapid urease test, morphology analysis and specific PCR (using primer VacA-F: 5′-GCCGATATGCAAATGAGCCGC-3′, VacA-R: 5′-CAATCGTGTGGGTTCTGGAGC-3′).

Hematoxylin-eosin staining and W-S silver staining. Samples were fixed in 10% formalin, dehydrated in ascending grades of ethanol solutions, cleared in xylene and then embed in paraffin wax. 4 μm thick sections of each sample were cut and mounted on glass slides. Tissue section then stained with hematoxylin and eosin for general histology.

For W-S silver staining, tissues embedded in paraffin were dehydrated, cleared and soaked in 1% silver nitrate for 30 min. Subsequently, samples were stained by staining solution consisting of 2% silver nitrate, 2% gelatin and 0.15% hydroquinone for 3–12 min. Sections were then washed by 56 °C sterile water, dehydrated by 95% ethanol solution and cleared in xylene. All the sections were observed under light microscopy and micrographs were collected at 100 magnification.

Statistical analysis. All data were expressed as mean ± S.D. and the results were statistically evaluated using students paired and unpaired *t*-test. $P < 0.05$ was considered as statistically significant difference. All data were analyzed by SPSS statistical version 22.0 software package (SPSS® Inc. USA).

3. Results

***H. pylori* infection upregulated expression of *CXCL-2*, *TLR-2* and downregulated expression of KSRP.** The 16S rDNA sequence phylogenetic tree shown that *H. pylori* SH1 was in a group with *Helicobacter pylori* strain PMSS1 (Fig. S1). *H. pylori* infection directly downregulated expression of KSRP (Figs. 1A and 3D), and upregulated expression of some inflammatory-related genes such as *CXCL-2*, *TLR-2* (Fig. 1B, D and 3E,H) both *in vitro* and *in vivo*. Yet expression of *HMOX-1* that triggered upregulation of interleukin 10, was not upregulated (Figs. 1C and 3I), suggesting that *HMOX-1* was not directly associated with *H. pylori* infection.

After *in vitro* transfection for 24 h, KSRP relative mRNA and protein expression were upregulated significantly compared to control (Fig. 1E and H). Once co-incubating with *H. pylori* for 12 h after transfection, KSRP relative mRNA and protein expression decreased dramatically (Fig. 1E and H), indicating that, KSRP played a role in *H. pylori*-induced innate immune response. Similar outcomes were also observed when co-incubate with *H. pylori* for 24 h

Please cite this article in press as: N. Li, et al., Effects of the RNA-binding protein, KSRP, on innate immune response against *Helicobacter pylori* infection in mice, Biochemical and Biophysical Research Communications (2017), https://doi.org/10.1016/j.bbrc.2017.12.016