**Helicobacter pylori cytotoxin-associated gene A impairs the filtration barrier function of podocytes via p38 MAPK signaling pathway**

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**MATERIALS AND METHODS**

**Cell culture and experimental protocol.** The conditionally immortalized mouse podocyte cell line was kindly provided by Dr Peter Mundel (Mount Sinai School of Medicine, New York, NY, USA) and cultured as described previously (Jing et al., 2015). Recombinant CagA protein which originates from Hp strain ATCC 43504 and contains three EPIYA motifs, was purchased from Shanghai Linc- Bio Science Co. Ltd (Shanghai, China). For dose-dependent experiments, differentiated podocytes were incubated with different concentration (0, 5, 10 and 20 μg/ml) of recombinant CagA protein for 72 h; for time-dependent experiments, cells were incubated with the optimal concentration of recombinant CagA protein for 12, 24, 48 and 72 h. Then the optimal concentration and incubation time were used in the subsequent experiments. To investigate the role of p38 MAPK signaling pathway in podocyte injury, differentiated podocytes were pretreated with 5 μM SB203580, a p38 inhibitor (ApexBio, TX, USA) for 1 h prior to being incubated with recombinant CagA protein.

**CCK-8 assay.** Cell viability was evaluated with CCK-8 assay (Signalway Antibody, MA, USA). 3000 cells per
well were plated in 96-well plate, cultured until attachment, then treated with different concentration (0, 5, 10 and 20 μg/ml) of recombinant CagA protein. At 0 h or 72 h of the treatment, 10 μl of CCK-8 solution was added to each well of the 96-well plate and the plate was incubated for 1 h at 37°C. Then the absorbance was measured at 450 nm using a spectrophotometer.

**Real time RT-PCR.** Total RNA was extracted from cells using Trizol reagent (Invitrogen, CA, USA). cDNA synthesis from total RNA was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific Fermentas, MA, USA). Resulting cDNA (1 μg) was amplified in real time, in 25 μl reaction mixture containing SYBR Green PCR Master Mix (Applied Biosystems, CA, USA), appropriate primer pairs and water using ABI 7300 thermocycler (Applied Biosystems, CA, USA). The reaction mixtures were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. The expression of target gene was normalized to the reference gene GAPDH in the same cDNA sample. The following primers were used: ZO-1: forward, 5'-AGGCTACCTTTGTATTCTC-3', reverse, 5'-TAGGGCACAGTATTGTATC-3'; p38 MAPK: forward, 5'-GTGGTTCACACCCGCAAGGTC-3', reverse, 5'-CGGTACGGTTCTGGCACTTC-3'; GAPDH: forward, 5'-ATCACGTGCCACCCGAGAAG-3', reverse, 5'-TCCAGAAGGACACATTG-3'. The results were analyzed using 2^(-ΔΔCT) method.

**Western blotting.** Total and membrane proteins were extracted using RIPA buffer (Solarbio, Beijing, China) and the membrane protein extraction kit (Beyotime, Nanjing, China), according to the manufacturer's instructions, respectively. Then the extracted proteins were resolved using SDS-PAGE and transferred to nitrocellulose (NC) membranes (Millipore, MA, USA). The NC membranes were blocked with 5% non-fat milk and incubated with primary antibodies overnight, followed by incubation with appropriate secondary antibodies for 1 hour at room temperature. The images were visualized using an enhanced chemiluminescence (ECL) detection system (Millipore, MA, USA). The primary antibodies used were as follow: anti-ZO-1 (1:50; Abcam, MA, USA), anti-p38 (1:1000; Cell Signaling Technology, USA), anti-p-p38 (1:1000; Cell Signaling Technology, MA, USA), anti-β-actin (1:1500; Cell Signaling Technology, MA, USA), and anti-GAPDH (1:1500; Cell Signaling Technology, MA, USA).

**Statistical analysis.** All the experiments were repeated at least three times. All data were presented as mean ± S.D. Differences between groups were evaluated by independent Student’s t-test using SPSS version 16.0 (SPSS Inc., USA). P<0.05 was considered significant.

**RESULTS**

**Dose- and time-dependent effect of CagA on the protein expression of ZO-1 in mouse podocytes**

Within the range of 5–20 μg/ml, CCK-8 assay demonstrated that CagA had no significant influence on cell viability in mouse podocytes (Fig. 1B). However, CagA decreased the protein expression of ZO-1 in mouse podocytes in a dose-dependent manner (Fig. 2A and B). Accordingly, 20 μg/ml was chosen as the optimal concentration of CagA for subsequent studies. When compared to the control group, CagA treatment decreased the protein expression of ZO-1 in a time-dependent manner.

**Albumin influx assay.** To evaluate the filtration barrier function of podocytes, albumin influx assay was performed as described previously (Kumar et al., 2010). Briefly, differentiated podocytes (5×10^6) were seeded onto the collagen-coated transwell filters (0.4-μm pore; Corning, NY, USA) at the top chamber. Then podocytes were serum-starved overnight (for the cell confluency level before CagA treatment see Fig. 1A) and treated with 20 μg/ml of recombinant CagA protein for 72 h. To determine the role of p38 MAPK signaling pathway in the filtration barrier function of podocytes, differentiated podocytes were pretreated with 5 μM SB203580 for 1 h prior to being treated with recombinant CagA protein. After treatment with recombinant CagA protein, podocytes were washed twice with PBS supplemented with 1 mM MgCl₂ and 1 mM CaCl₂ to preserve the cadherin-based junctions. Then the top chamber was refilled with 0.15 ml of RPMI 1640 and the bottom chamber with 1 ml of RPMI 1640 supplemented with 40 mg/ml of bovine serum albumin. The podocytes were incubated at 37°C for 1 h. Then a small aliquot of medium from top chamber was collected and the albumin concentration was measured using the bicinchoninic acid protein assay kit (Nanjing Jiansheng Bioengineering Institute, Nanjing, China).

**Figure 1. Cell confluency level and CCK-8 assay results.** (A) Podocytes at 100% confluence before CagA treatment photographed under an inverted microscope. Original magnification, ×100. (B) At 0 h or 72 h, CCK-8 assay showed that there was no significant difference in cell viability between podocytes treated with different concentration (0, 5, 10 and 20 μg/ml) of recombinant CagA protein.
The role of CagA in podocyte injury

Influence of CagA on the expression of total and membrane ZO-1 as well as on the filtration barrier function of mouse podocytes

In comparison to the control group, the mRNA expression of ZO-1 was significantly decreased after CagA treatment for 72 h (Fig. 3A). Consistently, with the mRNA change the protein levels of total and membrane ZO-1 were also downregulated (Fig. 3B and C). Moreover, albumin influx assay was used to evaluate the filtration barrier function of mouse podocytes. CagA treatment resulted in an increased albumin influx across the podocyte monolayer at 72 h, which suggested an impaired filtration barrier function of podocytes (Fig. 3D).

Effect of CagA on p38 MAPK signaling pathway in mouse podocytes

When compared to the control group, the level of p38 MAPK mRNA was significantly increased after CagA treatment for 72 h, while SB203580 pretreatment partly diminished this effect (Fig. 4A). Consistently with the mRNA level change, the protein levels of total and phosphorylated (activated) p38 MAPK were also increased after CagA treatment for 72 h, while SB203580 pretreatment partly prevented these changes (Fig. 4B, C and D).

Impact of p38 MAPK inhibition on the expression of ZO-1 and filtration barrier function of mouse podocytes incubated with CagA

Selective p38 MAPK inhibition via SB203580 pretreatment significantly increased the mRNA level of ZO-1 which was suppressed by CagA treatment, although the level was still statistically different from the control group (Fig. 5A). Similar results were observed for the ZO-1 protein expression (Fig. 5B and C). Moreover, albumin influx assay showed that SB203580 pretreatment notably attenuated albumin influx across the podocyte monolayer which was induced by CagA treatment, although it was still statistically different from the control group (Fig. 5D).

DISCUSSION

Among the virulence factors of Hp, CagA remains the only one which translocates into cells via type IV secretion system (Jimenez-Soto et al., 2009; Odenbreit et al., 2000; Song et al., 2013). For the mechanisms of CagA delivery, recent study revealed that after being exposed on the bacterial surface via type IV secretion, CagA interacts with phosphatidylserine of host plasma membrane and then enters the host cells via an endocytic process distinct from known endocytic pathways (Murata-Kamiya et al., 2010). CagA was also found in serum-derived exosomes in patients with Hp infection, and CagA-containing exosomes can be internalized into cells via endocytosis or membrane fusion, independently of type IV secretion (Shimoda et al., 2016). Therefore, it is acceptable that, by directly interacting with phosphatidylserine of host plasma membrane, exogenous CagA can enter the cells via endocytosis. Actually, similarly to the present study, previous in vitro studies showed that recombinant CagA protein may directly influence cell viability or function (Gajewski et al., 2015; Lin et al., 2015; Wang et al., 2016).

CagA can regulate the expression of target proteins in a dose- and/or time-dependent manner (Brandt et al., 2005; Kang et al., 2013; Lina et al., 2013). However, both...
unchanged and decreased ZO-1 expression were reported in gastric epithelial cells with CagA-positive Hp infection (Krueger et al., 2007; Zhang et al., 2014). While CagA-positive Hp infection activated β-catenin signaling in gastric epithelial cells, β-catenin inhibited the expression of ZO-1 in podocytes (Murata-Kamiya, 2011; Zhou et al., 2015). Therefore, it seems reasonable that CagA may have the potential to suppress ZO-1 expression via β-catenin. In the present study, we found that CagA could dose- and time-dependently decrease ZO-1 expression in mouse podocytes.

Tight junction protein ZO-1 located in the membrane surface of podocytes and plays an essential role in establishing podocyte filtration barrier (Ha, 2013; Itoh et al., 2014). Abnormal expression and/or distribution of ZO-1 may impair the filtration barrier function of podocytes and then induce proteinuria. In MWF rats, an animal model of spontaneous proteinuria, abnormal glomerular ZO-1 distribution alone may cause proteinuria (Ha, 2013). In animal model of diabetic kidney disease, high glucose decreases the expression and simultaneously alters the distribution of ZO-1 in glomerular epithelial cells, which is associated with proteinuria (Rincon-Choles et al., 2006). In the present study, we demonstrated that after CagA treatment, consistently with the reduced expression of total ZO-1, the distribution of membrane ZO-1 was also decreased, which consequently induced an increased albumin influx across the podocyte monolayer, resulting in an impaired filtration barrier function of mouse podocytes.

The p38 MAPK signaling pathway plays a crucial role in regulating cellular response (Cuadrado & Nebreda, 2010). CagA-positive Hp infection or CagA transfection may induce p38 MAPK phosphorylation in gastric epithelial cells (Allison et al., 2009; Liu et al., 2012). In Hp-dependent gastric mucosa-associated lymphoid tissue (MALT) lymphoma, the expression level of CagA was closely associated with the activation of p38 MAPK (Kuo et al., 2015). In the present study, we also revealed that CagA promoted the expression and phosphorylation of p38 MAPK in mouse podocytes. On the other hand, consistently with the previous reports, SB203580 pretreatment significantly inhibited these changes (Hai-Yan et al., 2013; Ding et al., 2015).

P38 MAPK activation mediated calcium oxalate crystal-induced downregulation of ZO-1 in distal renal tubular epithelial cells and regulated the distribution of ZO-1 in cell-cell contacts of keratinocytes (Peerapen & Thongboonkerd, 2013; Minakami et al., 2015). Both in human podocyte injury diseases and animal models of nephropathy, podocyte p38 MAPK signaling was activated and associated with proteinuria. P38 MAPK inhibition significantly ameliorated podocyte injury and suppressed proteinuria (Koshikawa et al., 2005). In the present study, we revealed that p38 MAPK inhibition by SB203580 pretreatment markedly improved CagA-induced downregulation of ZO-1 and consequently protected the filtration barrier function of mouse podocytes.

In summary, our results suggest that CagA, one of the key virulence factors of Hp, decreases the expression and membrane distribution of tight junction protein ZO-1, impairs the filtration barrier function of podocytes, while activating p38 MAPK signaling pathway in these cells. Selective p38 MAPK inhibition partly prevents CagA-induced filtration barrier dysfunction of podocytes through ameliorating ZO-1 downregulation. Therefore, CagA, at least via p38 MAPK signaling pathway, may induce podocyte injury. Anti-Hp therapy may be beneficial for the treatment of kidney diseases related to Hp antigen deposition.

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