CagA promotes proliferation and secretion of extracellular matrix by inhibiting signaling pathway of apoptosis in rat glomerular mesangial cells

Li Wang*, Rui-Zhi Tan*, Yue Chen, Hong-Lian Wang, Yu-Hang Liu, Dan Wen and Jun-Ming Fan

ABSTRACT
Cytotoxin-associated antigen A (CagA), a major virulence factor of Helicobacter pylori (Hp), is associated with the pathogenesis of peptic ulcer and gastric cancer. Recent researches demonstrated that Hp exists in palatine tonsil in all studied IgA nephropathy (IgAN) patients, most of which were CagA-positive, suggesting that CagA may be a causative pathogenic factor of IgAN. However, the underlying molecular mechanisms and signaling pathway are still largely unclear. In the present study, CCK8 assay, enzyme-linked immunosorbent assay, and immunohistochemistry were performed to investigate the effect of CagA on cell proliferation and extracellular matrix secretion in rat glomerular mesangial cells. RT-PCR and western blotting were used to reveal the potential signaling pathway. Rat glomerular mesangial cells were treated with recombinant CagA protein for 72 h, in a dose- and time-dependent manner. We found that CagA promoted cell proliferation and extracellular matrix secretion by inhibiting signaling pathway of apoptosis. Taken together, these findings suggested that CagA induced cellular injury in glomerular mesangium by proliferation and secretion of extracellular matrix, and may play an important role in pathogenesis of IgAN.

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Introduction
Immunoglobulin A nephropathy (IgAN), one of the most common glomerulonephritis worldwide, characterized by predominant IgA deposits and extracellular matrix deposition in the glomerular mesangium, is a major cause of end-stage renal disease. Recently, according to clinical and basic research data, multiple-hit hypothesis have been proposed about the pathogenesis of IgA nephropathy. One of them is abnormal mucosal immunity. IgA1 deposition in glomerular mesangium is present mostly in mucosal-type, which form polymeric immune complexes. Moreover, IgAN often aggravates or relapses during or after an upper respiratory infection, suggesting that mucosal infection may play a major role in the pathogenesis of IgAN.

Helicobacter pylori (Hp), a gram-negative bacterium, transmitted by either the fecal–oral or the oral–oral route, has been confirmed in the pathogenesis of chronic gastritis and peptic ulcer disease, also is a risk factor for gastric cancer. Previous research indicated that the antigen of Hp may be the susceptive antigen in membranous nephropathy. A recent study from Japan reported that Hp exists in palatine tonsil with coccoid form, and all of the patients with IgAN had Hp infection in palatine tonsil, presuming that immune complexes were formed by anti-Hp IgA antibody in palatine tonsil, and deposited in glomerular mesangium through blood circulation, suggesting that Hp may partially associated with pathogenesis of IgAN.

Cytotoxin-associated antigen A (CagA) is a virulence factors for Hp-inducing infection, and encoded by cagA. CagA is delivered into gastric epithelial cells after bacterial adherence via the bacterial type IV secretion system, and result in pathogenesis of peptic ulcer and gastric cancer. Notably, CagA was observed in 88.4% of tonsillar Hp-positive patients with IgAN. Furthermore, recent report showed that CagA destroys the gastric epithelial structure by mucosal inflammation. Moreover, CagA promoted the under glycosylation of IgA1 by down regulating of β1,3-
galactosyltransferase (C1GALT1) and its chaperone, which may participate in the pathogenesis of IgAN. All the above data suggested that CagA from tonsillar Hp infection may be a causative pathopoiesis of IgAN. However, the molecular mechanisms of CagA in pathogenesis of IgAN remain unclear.

In the present study, to determine the mechanism of CagA on IgAN pathogenesis, we investigated the effects of CagA on cell proliferation and secretion of extracellular matrix in rat glomerular mesangial cells, and tried to reveal the molecular mechanisms and signaling pathway involved in these effects by RT-PCR and western blotting.

Materials and methods

Cell culture

Rat glomerular mesangial cell line was a gift from Professor Qin Zhou (Chongqing medical university, Chongqing, China) and cultured in DMEM medium (Gibco, Los Angeles, CA) with 10% fetal calf serum (heat-inactivated at 56°C), 0.1 mg/mL streptomycin and 100 U/mL penicillin at 37°C with 5% CO2.

Drug treatments

For dose-dependent tests, cells were treated with 1, 2, and 4 µg/mL of recombinant CagA protein (LincBio Science, Shanghai, China) for 24 h, respectively. For time-dependent tests, cells were incubated with 4 µg/mL of recombinant CagA protein for 24, 48, and 72h, respectively. Cells treated with IL-1 serve as positive control, and PBS as the negative control.

Cell proliferation assay

The cell proliferation was analyzed by cell counting kit 8 (CCK8) assay (SaiChi Biotech, Beijing, China) according to the manufacturer’s instructions. 100 µL of cell suspension (5000 cells/well) was dispensed in a 96-well plate. The plate was pre-incubated for 24 h at 37°C in 5% CO2 air condition. The medium containing CagA or IL-1 of indicated concentration was refreshed and further incubated for 48 h in the incubator. 10 µL of CCK-8 solution was added to each well of the plate and the plate was incubated for 4 h. The absorbance at 450 nm was measures by microplate reader.

Enzyme-linked immunosorbent assay

ELISA was performed to detect the production of collagen I and collagen III in the supernatant from each well. Rabbit anti-rat collagen I and collagen III antibody (Diluted 1:100; Abcam, Cambridge, UK) was added into each well overnight at 4°C. After blocking, samples were added and cultured at 37°C for 3 h, and then incubated with horseradish peroxidase-conjugated rabbit anti-rat collagen I and collagen III antibody (Diluted 1:8000, Abcam, Cambridge, UK) at 37°C for 1 h. The color was developed by tetramethyl benzidine dilution (TMB) and quantification was determined by measuring absorbance at 450 nm using a microplate reader (Infinite M200, TECAN, Mönndorf, Switzerland).

RT–PCR assay

Total RNA was harvested from rat glomerular mesangial cells using Trizol reagent (Invitrogen, Carlsbad, CA). For the cDNA synthesis, total RNA was reverse-transcript using the RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania). PCR amplification was performed at 95°C for 2 min followed by 30 cycles of 30 s denaturation at 94°C, 30 s annealing at 60°C and 30 s extension at 72°C. The PCR reaction was performed using the following primers: BAX: forward, 5’-GCA AACTGGTG CTCAAGG-3’, reverse, 5’-GTCCGAAAGT AGGAAAGG-3’; BCL-2: forward, 5’-GGCATCTTTCTCT TCCAGC-3’, reverse, 5’-ATCCCCAGCTCGTTATCC-3’; Collagen I: forward, 5’-TGGAGACAGGTCAAGCTC-3’, reverse, 5’-TATTCGA TGACTGTCTTGCC-3’; Collagen III: forward, 5’-TAAAAGGTTGAACGAGGCAG T-3’, reverse, 5’-ACGTCCCCATTATGGCCAC-3’; GAPDH: forward, 5’-AC CACA GTCCATGCGCATC-3’, reverse, 5’-TCCA CACCGCTGTGCTGA-3’. The product of RT-PCR was separated by 1% agarose gel. The results were analyzed by using Gel-pro Analyzer Software (Version 4.0, Media Cybernetics, Bethesda, MD). The expression of target gene was normalized to the reference gene GAPDH.

Western blotting

The protocol of Western blotting was described previously. The primary antibodies used in this study were rabbit anti-collagen I and collagen III antibody (Diluted 1:1000, Abcam, Cambridge, UK), rabbit anti-BAX antibody (Diluted 1:1000, Abcam, Cambridge, UK), and rabbit anti-BCL-2 antibody (Diluted 1:1000, Abcam, Cambridge, UK). The secondary antibodies were horse-radish peroxidase-conjugated secondary anti-rabbit antibodies (1:3000; ZSGB-Bio, Beijing, China). Total protein (25 µg) was resolved on a 10% SDS-PAGE gel (Solarbio, Beijing, China) and transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA). Then, the blot was probed with the indicated primary antibodies at 4°C overnight, followed by incubation with the corresponding secondary antibody at room temperature.
temperature for 1 h. Finally, the signal was developed using Immobilon Western Chemiluminescent HRP substrate (Millipore, Etobicoke, Canada) and exposed to X-ray film (Kodak, Rochester, NY). The relative protein levels of collagen I, collagen III, BAX, and BCL-2 were normalized to GAPDH.

**Immunohistochemistry**

Rat glomerular mesangial cells were treated with 10 ng/mL IL-1 and 4 μg/mL CagA at 37°C in 5% CO₂ air condition for 72 h, were grown on glass coverslips with a diameter of 13 mm for immunostaining. Cells were washed by PBS for three times and fixed with 2% paraformaldehyde, then stained using the SABC kit (ZSGB-Bio, Beijing, China) following the manufacturer’s instructions. The primary antibodies were rabbit anti-rat PCNA antibody (Diluted 1:200, Abcam, Cambridge, UK).

**Statistical analysis**

Data are presented as means±SD. All results were analyzed using SPSS statistical software (Version 19.0; SPSS, Chicago, IL). p-Values less than 0.05 were considered significant.

**Results**

To investigate the proliferation in rat glomerular mesangial cells treated with CagA, we performed the CCK8 assay, by which we evaluated the effect of increased doses of CagA (from 1 to 4 μg/mL) on the proliferation of rat glomerular mesangial cells at different times of treatment (24–72 h) (Figure 1A). The treatment for 24–72 h with each concentration of CagA results in a progressive increase of cell proliferation. The proliferation of cells treated with 4 μg/mL CagA for 72 h was higher than that treated for 24 h and 48 h, also higher than that treated with other concentration. Considering the cytotoxicity of higher concentration of CagA, 4 μg/mL was chosen as the optimal concentration of CagA for further study, and according to the time-dependent experiments, we thought that cells were treated for 72 h was appropriate.

ELISA was performed to investigate the effect of CagA on secretion of extracellular matrix in rat glomerular mesangial cells. The 24–72 h exposure of cells to 1–4 μg/mL CagA resulted in progressive raise of collagen type I and collagen type III concentration (Figure 1B and C). The concentration of collagen type I and collagen type III were strongly increased in cells treated with 4 μg/mL CagA for 72 h, suggesting that CagA upregulates the secretion of extracellular matrix in rat glomerular mesangial cells.

Cells were exposed to control, 10 ng/mL IL-1 and 4 μg/mL CagA for 72 h, respectively. The CCK8 assay indicated that the proliferation of cells treated with 4 μg/mL CagA or IL-1 were increased identically (Figure 2A) and the results of Elisa showed that the concentration of secreted collagen type I and III by cells incubated with CagA and IL-1 were also upregulated (Figure 2B and C), suggesting the secretion of extracellular matrix was increased in these cells. These findings demonstrated
that CagA can increases cell proliferation and secretion of extracellular matrix in rat glomerular mesangial cells, which were consistent with IL-1.

Immunohistochemistry analysis was performed to investigate the expression of PCNA in rat glomerular mesangial cells. The results showed that the expression of PCNA in cells treated with IL-1 and CagA were largely increased (Figure 3A). It was further validated by semiquantitative analysis of the staining signal in Figure 3(A and B).

RT-PCR were performed to detect the mRNA expression levels of collagen type I, collagen type III, BAX, and BCL-2 in rat glomerular mesangial cells treated with CagA (Figure 4A). Compared with control, the mRNA expression of collagen type III was significantly upregulated by CagA. And this effect was stronger than that of IL-1 (Figure 4B). Meanwhile, compared with control, the mRNA expression of collagen type I was also increased by CagA, although the effect was weaker than that of IL-1 (Figure 4C). However, compared with control, the mRNA expression of BAX was downregulated by CagA, and the effect was stronger than that of IL-1 (Figure 4D). Consistent with IL-1, CagA upregulated the mRNA expression of BCL-2 in rat glomerular mesangial cells (Figure 4E).

Western blotting were performed to assess the protein expression levels of collagen type I, collagen type III, BAX, and BCL-2 in rat glomerular mesangial cells exposed to CagA (Figure 5A). Compared with control, the protein expression of collagen type III, collagen type I, and BCL-2 were upregulated by CagA in rat glomerular mesangial cells, which were consistent with IL-1. However, compared with control, the protein expression of BAX was reduced by CagA in cells.

**Discussion**

In the present study, we revealed that CagA acted as a major virulence factor of Hp, promoting cell proliferation and extracellular matrix secretion in rat glomerular mesangial cells. The molecular mechanisms for these effects, is at least partially due to the increased expression of Bcl-2, which is known as an apoptosis inhibiting gene.

The diagnostic hallmark of IgA nephropathy is the IgA deposits, which are immune complexes that mainly consist of IgA1 in dimer or polymer form. Normally, IgA1 dimer is generated by mucosal tissue, suggesting that IgA1 deposited in glomerular mesangium may be produced by extra-renal mucosal tissue via antigen stimulation. Thus, immune dysfunction in mucosal tissue, such as palatine tonsil, has been considered to be the important research hot spot in pathogenesis of IgAN. Notably, most patients with IgAN are Hp-positive in palatine tonsil, and about 88% Hp-positive patients are CagA-positive, implying that CagA may be associated with IgAN pathogenesis.

Abnormal proliferation in glomerular mesangium is an important pathological basis for development of IgAN. Previous research reported that interleukin-1 (IL-1) increased cell proliferation and extracellular matrix secretion in rat glomerular mesangial cells in vitro. Therefore, in the present study, IL-1 acted as a positive control. We found that CagA induced cell proliferation in rat glomerular mesangial cells in dose-dependent (1–4 μg/mL) and time-dependent (24–72 h) manner, which is similar to IL-1. Furthermore, the results of immunohistochemistry analysis for PCNA showed that the expression of PCNA in cells treated with IL-1 and CagA were significantly upregulated, which is consistent
Figure 4. Effects of CagA on mRNA expression of Collagen type I, Collagen type III, BAX, and BCL-2 in rat glomerular mesangial cells. Cells were incubated with 10 ng/mL IL-1 and 4 μg/mL CagA for 72 h, respectively. (A) The mRNA expression of Collagen type I, Collagen type III, BAX, and BCL-2 in CagA and IL-1 treated cells were measured by RT-PCR. (B) Compared with negative control, CagA upregulated the mRNA expression of Collagen type III, and the effect was stronger than that of IL-1. (C) Compared with negative control, CagA upregulated the mRNA expression of Collagen type I, although the effect was weaker than that of IL-1. (D) Compared with negative control, CagA significantly downregulated the mRNA expression of BAX, although the effect was a little weaker than that of IL-1. (E) Compared with negative control, CagA upregulated the mRNA expression of BCL-2. *p<0.05 versus treatment with control. **p<0.01 versus treatment with control.

Figure 3. CagA increases the level of proliferating cell nuclear antigen (PCNA) in rat glomerular mesangial cells. (A) Immunohistochemical analysis of PCNA in rat glomerular mesangial cells after exposing to 10 ng/mL IL-1 and 4 μg/mL CagA for 72 h. Bar indicates the same magnification (200×). (B) Compared with negative control, CagA and IL-1 significantly increased the expression of PCNA in rat glomerular mesangial cells. The value of IOD in (A) was measured by Image-Pro Plus software (Version 6.0, Media Cybernetics, Bethesda, MD). *p<0.05 versus treatment with control. **p<0.01 versus treatment with control.
with CCK8 assay. PCNA, proliferating cell nuclear antigen, expresses from G1 phase, and reaches the highest level of expression in S phase, whose expression level can represents the degree of proliferation. On the other hand, when IgAN occurred, secretion of collagen type I and III were promoted, that stimulated cells to secrete extracellular matrix which subsequently aggravated the injury of kidney. In the present study, CagA increased extracellular matrix secretion in rat glomerular mesangial cells in dose-dependent (1–4 μg/mL) and time-dependent (24–72 h) manner, which is consistent with IL-1. These results strongly suggested that CagA may be involved in IgAN pathogenesis and development.

Notably, the previous studies almost focused on the pathological and physiological changes caused by CagA. However, the underlying molecular mechanisms of proliferation and secretion of extracellular matrix stimulated by CagA remain to be elucidated. Previous study demonstrated that renal glomerular disease were at least partially associated with apoptosis. Bcl-2 is an anti-apoptotic protein, whose homologous proteins contain BAX, Bcl-XL, BAD, etc. Bcl-2 can inhibit apoptosis by preventing mitochondria to release apoptotic proteins, suppressing activation of cysteine protease and blocking peroxidation of lipid. In the present study, the mRNA and protein expression of Bcl-2 were increased in CagA-stimulated cells, and expression of BAX were decreased in CagA-treated cells, suggesting that CagA upregulated the expression of Bcl-2 to inhibit apoptosis, and downregulate the expression of BAX to promote proliferation, subsequently leading to insufficient apoptosis and excessive proliferation in glomerular mesangial cells.

In conclusion, we revealed that CagA, a major virulence factor of Hp, increased cell proliferation and extracellular matrix secretion in rat glomerular mesangial cells. Moreover, CagA induced excessive proliferation in glomerular mesangial cells, at least partially by increasing the expression of Bcl-2 and reducing the expression of BAX. On the other hand, CagA promoted the secretion of extracellular matrix, may through increasing the expression of Collagen type I and III. In the present study, the results suggested that CagA is strongly associated with pathogenesis of IgAN, and anti-Hp or anti-CagA therapy may be a potential treatment for IgAN.

**Disclosure statement**

The authors report no conflicts of interest.
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References

1. D’Amico G. The commonest glomerulonephritis in the world: IgA nephropathy. Q J Med. 1987;64(245):709–727.
2. Kiryluk K, Novak J. The genetics and immunobiology of IgA nephropathy. J Clin Invest. 2014;124(6):2325–2332.
3. Suzuki H, Kiryluk K, Novak J, et al. The pathophysiology of IgA nephropathy. J Am Soc Nephrol. 2011;22(10):1795–1803.
4. Lai KN. Pathogenesis of IgA nephropathy. Nat Rev Nephrol. 2012;8(5):275–283.
5. Boyd JK, Cheung CK, Molyneux K, Feehally J, Barratt J. An update on the pathogenesis and treatment of IgA nephropathy. Kidney Int. 2012;81(9):833–843.
6. Eusebi LH, Zagari RM, Bazzoli F. Epidemiology of Helicobacter pylori infection. Helicobacter. 2014;19(Suppl1):1–5.
7. Rauws EA, Tytgat GN. Cure of duodenal ulcer associated with eradication of Helicobacter pylori. Lancet. 1990;335(8700):1233–1235.
8. Wroblewski LE, Peek, Jr RM, Wilson KT. Helicobacter pylori and gastric cancer: Factors that modulate disease risk. Clin Microbiol Rev. 2010;23(4):713–739.
9. Nagashima R, Maeda K, Yuda F, Kudo K, Saitoh M, Takahashi T. Helicobacter pylori antigen in the glomeruli of patients with membranous nephropathy. Virchows Arch. 1997;431(4):235–239.
10. Kusano K, Tokunaga O, Ando T, Inokuchi A. Helicobacter pylori in the palatine tonsils of patients with IgA nephropathy compared with those of patients with recurrent pharyngotonsillitis. Hum Pathol. 2007;38(12):1788–1797.
11. Gobert AP, Asim M, Piazuelo MB, et al. Disruption of nitric oxide signaling by Helicobacter pylori results in enhanced inflammation by inhibition of heme oxygenase-1. J Immunol. 2011;187(10):5370–5379.
12. Segal ED, Cha J, Lo J, Falkow S, Tompkins LS. Altered states: Involvement of phosphorylated CagA in the induction of host cellular growth changes by Helicobacter pylori. Proc Natl Acad Sci USA. 1999;96(25):14559–14564.
13. Ferreira RM, Machado JC, Figueiredo C. Clinical relevance of Helicobacter pylori vacA and cagA genotypes in gastric carcinoma. Best Pract Res Clin Gastroenterol. 2014;28(6):1003–1015.
14. Kusano K, Inokuchi A, Fujimoto K, et al. Coccoid Helicobacter pylori exists in the palatine tonsils of patients with IgA nephropathy. J Gastroenterol. 2010;45(4):406–412.
15. Amieva MR, Vogelmann R, Covacci A, Tompkins LS, Nelson WJ, Falkow S. Disruption of the epithelial apical-junctional complex by Helicobacter pylori CagA. Science. 2003;300(5624):1430–1434.
16. Yang M, Li FG, Xie XS, Wang SQ, Fan JM. CagA, a major virulence factor of Helicobacter pylori, promotes the production and underglycosylation of IgA1 in DAKKI cells. Biochem Biophys Res Commun. 2014;444(2):276–281.
17. Mao N, Cheng Y, Shi XL, et al. Ginsenoside Rg1 protects mouse podocytes from aldosterone-induced injury in vitro. Acta Pharmacol Sin. 2014;35(4):513–522.
18. Wyatt RJ, Julian BA. IgA nephropathy. N Engl J Med. 2013;368(25):2402–2414.
19. Yu HH, Chu KH, Yang YH, et al. Genetics and immuno-pathogenesis of IgA nephropathy. Clin Rev Allergy Immunol. 2011;41(2):198–213.
20. Coppo R. Can a dysregulated mucosal immune system in IgA nephropathy be controlled by tonsillectomy? Nephrol Dial Transplant. 2010;25(8):2395–2397.
21. Barratt J, Bailey EM, Buck KS, et al. Exaggerated systemic antibody response to mucosal Helicobacter pylori infection in IgA nephropathy. Am J Kidney Dis. 1999;33(6):1049–1057.
22. Wang R, Wan Q, Zhang Y, et al. Emodin suppresses interleukin-1 beta induced mesangial cells proliferation and extracellular matrix production via inhibiting P38 MAPK. Life Sci. 2007;80(26):2481–2488.
23. Zhang W, Khanna P, Chan LL, Campbell G, Ansari NH. Diabetes-induced apoptosis in rat kidney. Biochem Mol Med. 1997;61(1):58–62.
24. Uda S, Yoshimura A, Sugenooya Y, Inui K, Taira T, Ideura T. Mesangial proliferative nephritis in man is associated with increased expression of the cell survival factor, Bcl-2. Am J Nephrol. 1998;18(4):291–295.