Original Article

Secreted Protein HP1286 of *Helicobacter pylori* Strain 26695 Induces Apoptosis of AGS Cells*

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Abstract

Objective Secreted proteins of *Helicobacter pylori (H. pylori)* interact with gastric epithelium cells and may contribute to cell damage. Considering the fact that HP0175 and hypothetical conserved protein HP1286 are included in the group and that HP0175 is a well-known apoptosis-induced factor, the present study aims to clarify whether HP1286 plays a role in bacterial pathogenicity or even functions as an apoptosis-induced factor in human stomach.

Methods Two genes encoding HP1286 and HP0175 were cloned into pET32a vector and expressed as recombinant proteins in *Escherichia coli (E. coli)* BL21. Signal peptide sequences were not included. The recombinant proteins were purified with His SpinTrap and desalted by using HiTrap Desalting. Immunoreactivity of the proteins was determined by Western blot. Human gastric epithelial cell AGS was challenged with these endotoxin-free proteins; and apoptosis of cells was assayed with the Cell Death ELISA kit.

Results Recombinant proteins and their respective products whose N-terminal his-tag were removed with thrombin were recognized by serum from the patient infected with *H. pylori*. Apoptotic AGS cells challenged by HP1286 of *H. pylori* 26695 were four times more than untreated cells. In addition, apoptosis-induced ability of HP1286 of SS1 was not as strong as that of *H. pylori* 26695 strain.

Conclusion HP1286 of *H. pylori* 26695 induces apoptosis of AGS cells in a time-dependent manner, however the apoptosis-induced ability of HP1286 may differ due to variations between different strains.

Key words: *H. pylori*; HP1286; AGS; Apoptosis

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram-negative, noninvasive bacterium which colonizes in human stomach and causes mucosal inflammatory response and many gastric diseases, such as gastroduodenal ulcers, chronic gastritis and gastric cancer[1-3]. Upon infection with *H. pylori*, both proliferation and apoptosis in gastric epithelium increase but without mucosa thickening[4]. Although the apoptosis phenomenon exists in normal human stomach which

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contributes to physiological epithelial cell loss, *H. pylori* infection does increase the proportion of apoptotic epithelium cell from about 3% to 16%[5]. And the distribution of apoptotic cells in the stomach expands from superficial epithelium to the depth of glands. After eradication of *H. pylori*, apoptosis was found to have been decreased consistently[6]. *In vitro*, typical morphological changes of apoptosis were observed under transmission electron microscopy in human gastric adenocarcinoma SGC-7901 cells co-cultured with *H. pylori* strain NCTC 11637[7]. Also an unknown secreted ingredient from *H. pylori* was able to induce cell-cycle arrest in T lymphocytes[8]. As a result, both *H. pylori* bacteria and gradients could induce apoptosis of gastric cells.

Most phenomena suggested that apoptosis was a direct effect of *H. pylori* infection, rather than a subsequent response to inflammation[5]. And some ingredients, such as urease, HP0175 and Gamma-glutamyltranspeptidase (γ-ggt), have been identified as apoptosis-induced factors. Among these proteins, urease and HP0175 can induce apoptosis of Kato-III and AGS cells by interacting with Class II MHC (major histocompatibility complex) and TLR4 (Toll-like receptor) on cell surface respectively[9-10]. Conditionally, urease can only induce apoptosis of cells with Class II MHC expression[9]. So it seems that multiple components play roles in a ligand-receptor manner and the mechanism is complex.

Since secreted proteins interact with host cells directly *in vivo*, some components in this group may be candidates of apoptosis-inducing factors. In our previous experiments, hypothetical conserved protein with typical signal peptide HP1286[11-13] and secreted proteins HP0175 from *H. pylori* strain SS1 with sound colonizing ability in mouse stomach as clinic *H. pylori* strains in human stomach were found to have contact with mouse epithelium cells directly (unpublished data). HP0175 executes apoptosis of human gastric epithelial cell line AGS in a time and dose-dependent manner by interacting with TLR4. The effect is confirmed by the fact that apoptosis-induced ability would be impaired by anti-TLR4 mono-antibody or an isogenic mutant of *H. pylori* with disrupted HP0175 gene[10,14].

Taking into account former reports, we wondered whether protein HP1286 also was acting as one of apoptosis-inducing factors. HP1286 from two *H. pylori* strains SS1 and 26695 were expressed in *E. coli* BL21 as his-tagged fusion proteins. And human gastric epithelial cell line AGS was challenged with the purified proteins. His-tagged HP0175 was also expressed in *E. coli* and used as positive control.

METHODS

Culture of H. pylori and AGS Cells

Pure cultures of *H. pylori* strains 26695 and SS1 presented by Dr. H. Mitchell and Dr. Jani O'Rourke from the Culture Collection at the School of Microbiology and Immunology, University of New South Wales, were cultured on Columbia agar (Oxoid Ltd, Basingstoke, UK) containing sheep blood (5%, v/v). They were grown for 48 h at 37 °C in an atmosphere of 5% O_2, 85% N_2, and 10% CO_2. The human gastric epithelium cell lines AGS (purchased from ATCC) was maintained in RPMI 1640 (Gibco, Germany) supplemented with 10% heat-inactivated FBS (Gibco, Germany) at 37 °C in a humidified atmosphere containing 5% CO_2.

Cloning of Genes Encoding HP0175 and HP1286

Genes encoding HP0175 and HP1286 were amplified by PCR. Sequences for signal peptide, identified with SignalP 3.0 Server (http://www.bs.dtu.dk/services/SignalP/), were not included. Primers of HP0175 were the same as designed in the document[10]. They were 5'-GCTGGATCCGCAAATAAGCTACGCATAAC-3' (sense) and 5'-GTGAGCTCTTACTGTTGATAACAATTTTAG-3' (anti-sense) harboring endonuelease sites *BamH*I and *Sac*I respectively. Primers of HP1286 were: 5'-TCGGATCCAAACCTTAACGATTG-3' (sense) and 5'-CTGTCGACTTATTGGGCTAAGCTTC-3' (anti-sense) with *BamH*I and *Sal*I at both ends respectively. These PCR productions were ligated to pET32a expression vector. Inserted fragments were sequenced and the HP1286 segments from other *H. pylori* strains were compared by Vector NTI Suite 6.

Expression and Purification of HP0175 and HP1286

E. coli strain BL21 harboring the right recombinant plasmid was inoculated into LB liquid medium and induced for 20 h at 28 °C by adding 0.8 mmol/L IPTG at the beginning of logarithmic phase. Cells were harvested and lysed with BugBuster Protein Extraction Reagent (Novagen, USA) supplemented with 1 μL Benzonase (Novagen, USA) per mL. After incubation on the shaking platform for 20 min at room temperature, insoluble cell debris was removed by centrifugation at 16 000 g for

20 min at 4 °C. The supernatant was subjected to SDS-PAGE to confirm whether the target proteins were expressed in a soluble pattern or not.

His SpinTrap (GE Healthcare, USA) was used to purify the His-tagged target proteins. Briefly, the purification contains four steps: equilibration, sample application, washing and elution. Before adding eluent containing 500 mmol/L imidazole, elution buffer with 200 mmol/L imidazole was used to solubilize and remove contaminating proteins.

Imidazole in eluent was removed by using HiTrap Desalting (GE Healthcare, USA). All experimental procedures were carried out by following the instructions. In the final elution step, 20 mmol/L PBS was used to elute the His-tagged proteins.

The concentration of these purified proteins was determined with Bradford assay. Endotoxin contamination in these desalting proteins was assayed by using E-TOXATE Kits (Sigma-Aldrich, USA).

Immunoblot Analysis

Purified proteins were separated by SDS-PAGE and transferring onto nitrocellulose membrane in semi-dry condition. These membranes were blotted with 5% skim milk powder for 1 h at room temperature and then incubated with primary antibody (mouse anti-his mAb, Seajet Scientific Co. Ltd, Beijing, and serum from *H. pylori* infected patient) overnight at 4 °C. After washing for three times to remove residual primary antibody, the membranes were incubated with HRP conjugated secondary antibody (goat anti-mouse and goat anti-human antibody, Zhongshan Biotech Co. Ltd, Beijing) diluted with blocking buffer for 1 h at room temperature. Enhanced chemiluminescence (ECL, Amersham, UK) detection kits and X-films were used to visualize the reaction bands.

AGS Cells Challenged with Purified Proteins and Apoptosis Detection

About 24 h before being challenged with purified proteins, AGS cells were inoculated in 24-well plate at 1.5×10^3 cells /well, which made them 60%-70% confluent at the time of treatment. Medium was replaced with fresh culture medium supplemented with 150 ng/mL purified his-tagged proteins. Plates were returned to the incubator for different time periods. Apoptotic cells were determined with the Cell Death Detection ELISA kit (Roche, USA) according to the instructions. The experiment was duplicated for two times.

RESULTS

Purification of HP0175 and HP1286

In order to get purified HP1286 protein and study its potential apoptotic ability, his-tagged HP0175 and HP1286 without peptide signal were expressed in the soluble pattern. After washing with eluted buffer containing low concentration of imidazole, many contaminated proteins could be removed and the target proteins in the final eluent seemed pure (Figure 1). And by using small volume eluate to dissolve target proteins in the desalting step, the concentration of recombinant proteins increased.

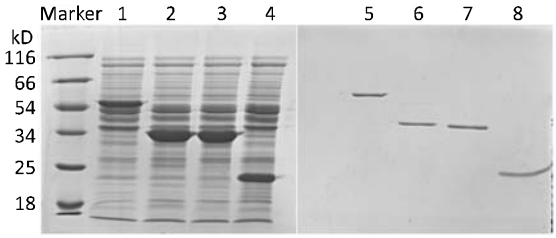

Figure 1. Purification of recombinant *H. pylori* proteins from *E. coli*. BL21. Marker: protein mass markers (Unstained Protein Molecular Weight Marker, #SM0431, Fermentas). Left: whole bacterial extraction of *E. coli* harboring different plasmids. 1: pET32a-HP0175, 2: pET32a-HP1286 (SS1), 3: pET32a-HP1286 (26695), 4: pET32a. Right: Elute for the purified bacterial extraction. 5: pET32a-HP0175, 6: pET32a-HP1286 (SS1), 7: pET32a-HP1286 (26695), 8: pET32a.

Immunoblot Analysis

Purified proteins and their products without N-terminal his-tag were assayed with western blot to confirm their accuracy. Western blots probed with anti-his antibody showed the exact position of HP0175 and HP1286 on gels. Fusion proteins and their products without tag were also recognized by serum from *H. pylori* infected patients (Figure 2). So these proteins were expressed well.

Assay for Endotoxin

For each sample, about 1 μg purified recombinant protein in desalting eluent was used to qualitatively determine the endotoxin level. At the end of incubation, the viscous or even clear liquid at the bottom of tubes could flow along the tube wall.

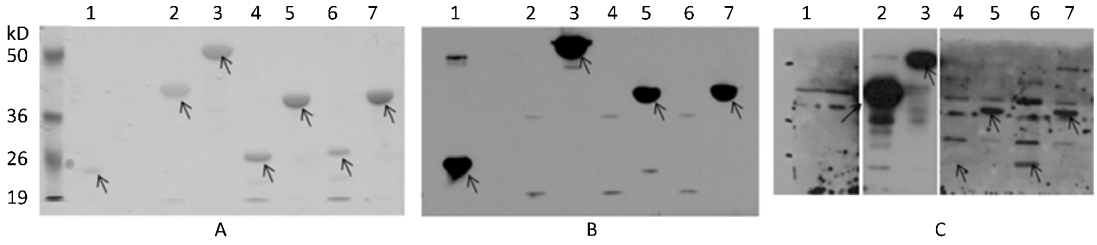

Figure 2. Western blot (WB) analysis of purified proteins. The arrows indicated their positions. 1: his-tagged PET 32a, 2: HP0175, 3: HP0175-his, 4: HP1286(26695), 5: HP1286-his(26695), 6: HP1286(SS1), 7: HP1286-his(SS1). (A) Coomassie Blue-stained gels of purified proteins and the proteins without N-terminal his tag by digesting with thrombin, (B) WB by mouse anti-his mono-antibody, (C) WB by serum from *H. pylori* carrier.

The results illustrated that each aliquot containing about 1 μg protein did not contain endotoxin or contained endotoxin at a level below the detection limit of assay, i.e. about 5-10 pg/μg protein[15]. Then the remaining endotoxin would not influence the following apoptosis detection.

Cell Apoptosis Detection

Cell death was assessed by quantitatively measuring histone-associated single- and mono-DNA fragments. As showed in Figure 3, His-tagged HP0175 was able to induce apoptosis of AGS cells in a time-dependent manner as ever reported. And his-tagged HP1286 from *H. pylori* 26695 was also able to induce apoptosis of AGS cells four times more than untreated cells, whereas HP1286 from SS1 did otherwise.

DNA variation between H. pylori Strain 26695 and SS1

Apoptosis-inducing abilities of HP1286 from *H. pylori* strains SS1 and 26695 were different. According to the sequencing results, there were 19 different amino acid sites between the two strains. And sequences of HP1286 from other four strains (from NCBI website) were also included. 18 of the 19 different amino acids in strains 26695 and SS1 were in accord with those in the other two strains. So it seemed that variations in some strains might be identical and follow some rules. The comparison was shown in Figure 4.

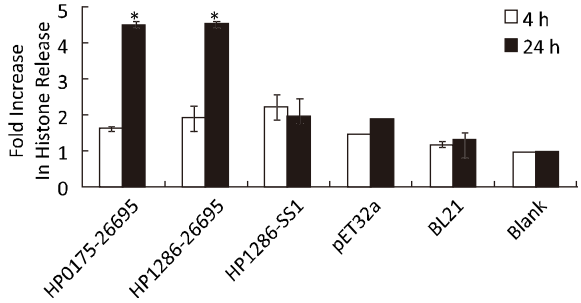

Figure 3. Purified proteins-induced apoptosis of AGS cells. Cells were incubated with purified protein (150 ng/mL) and equal amounts of His-tagged empty vector protein for various periods of time. AGS cells treated with equal volume elute from BL21 without any plasmid, which were purified with the same process as his-tagged proteins, were used as control. And cells without any treatment were used as blank. In each case, the medium containing different proteins was removed at the indicated time. Cells were washed and lysed, and cell death was measured with the Cell Death ELISA kit as described in Materials and Methods. Results are interpreted as the fold increase in the release of histone compared with that in the control (pET32a vector protein and elute from BL21 bacteria lysate treated AGS, untreated AGS); values are the mean±SD of three different experiments. $^{*}P<0.05$ *vs.* blank (by chi square test).

DISCUSSION

During the course of infection and colonization of *H. pylori* in human stomach, secreted proteins are the first constituents contacting host tissues and may mediate important interactions. In our former experiments, γ-glutamyltranspeptidase (ggt), secreted proteins HP1286 and HP0175 of *H. pylori* strain 26695 and SS1 were found to adhere to mouse stomach mucosa (data not shown). By interacting with gastric mucosa, HP0175 can induce apoptosis of AGS cells through TLR4 which are constitutively expressed on

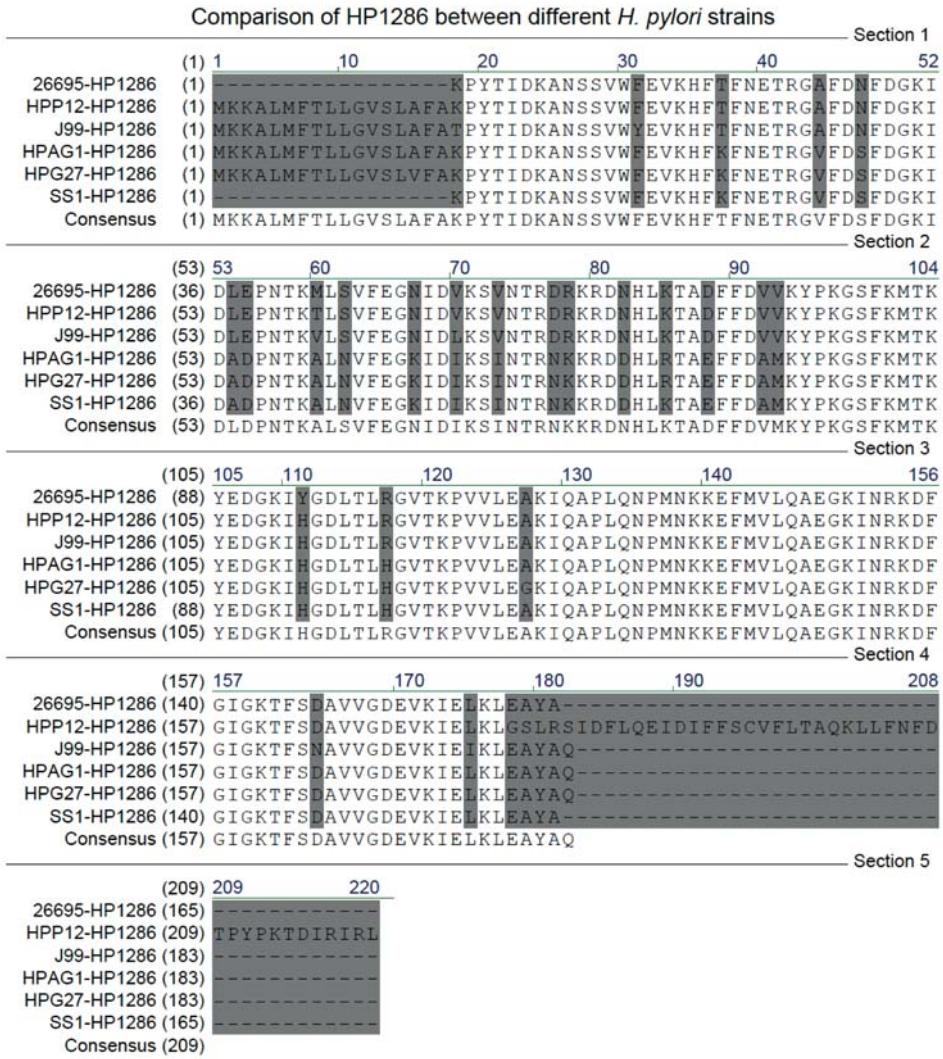

Figure 4. Schematic illustration of amino acid sequence divergence of HP1286 without signal peptide of *H. pylori* strains 26695 and SS1 and sequences of other strains downloaded from NCBI website. The difference was marked as dark background.

surface of cells[16] and γ-ggt can also induce mitochondria-mediated apoptosis in AGS cells, although the receptor remains unknown[17]. In order to study the role of HP1286, residues from 18 to 182 without signal peptide were cloned and expressed in BL21 in soluble pattern. The purified protein can be recognized by serum from HP carriers, indicating that the recombinant protein was immunoreactively positive. As positive control, HP0175 without signal peptide was prepared by the same way and the Western blot analysis indicated that HP0175 could also be recognized by serum of *H. pylori* carrier. So these proteins were expressed in the correct type.

Taking into account the established results, a question whether HP1286 had the apoptosis-inducing ability was first considered. Consequently, AGS cell was challenged with the purified HP1286 and HP0175 protein to evaluate the effect on human gastric epithelium cell. The concentration of purified proteins in medium was adjusted to a moderate level according to reports on HP0175. Their apoptosis-inducing ability was determined by detecting histone-associated DNA fragments in one immunoassay, which demonstrates occurrence of the internucleosomal degradation of genomic DNA during apoptosis of AGS cells. The results indicated that AGS challenged with HP1286 and HP0175 of 26695 experienced a time-dependent apoptosis.

The apoptosis of AGS cells was attributed to these proteins indeed. Firstly, abundant imidazole and salty ions were removed by using HiTrap Desalting. Secondly, the content of endotoxin in

purified proteins added into the medium of cultured cells was under the detection limit of limulus test, and this influencing factor could also be ruled out. Thirdly, apoptosis of AGS induced by pET32a vector protein with his-tag was milder obviously. In addition, HP1286 from *H. pylori* strain SS1 could not induce apoptosis as strong as 26695 did. According to DNA sequencing results, variations at the DNA level existed between the two stains. When compared with HP1286 amino sequence of four other strains published on NCBI website, it seemed that the variations may not be random. *H. pylori* strains 26695, HP_P12 and J99 were all from patients suffering from gastric inflammation and peptic ulcer. However, information of HPAG1 and HPG27 was not in detail. Therefore, the difference in some amino acids might contribute to their different apoptosis-inducing abilities. In future studies, we will sequence HP1286 gene of more *H. pylori* strains to confirm the interaction mechanism and choose some sites for mutants to verify the roles of these variations.

Apoptosis is an orchestrated suicide program, and is a key means to mediate homeostasis of gastric epithelium in physiological status[18]. However, an overall increase of apoptosis was observed and the distribution expanded to deeper gastric pits after *H. pylori* infection. As a candidate of apoptosis-inducing factor, how HP1286 transmits the signal from outside remains unknown. Structurally, HP1286 belongs to YceI-like protein family, which is characterized by the lipocalin fold. Also there is an inner cavity appropriate for a roughly linear chain of about 22 carbon atoms[19]. Such information hints the clue of ligand of HP1286. Interestingly, two of the above variations were just in the inner cavity. Thus, if the ligand was related with transmitting apoptotic signals, variations might be partially responsible for the different apoptotic abilities. In further research, we may use immunoprecipitation or pull-down techniques to search for its exact ligand and study which classical factors responsible for apoptosis are included in its downstream pathway. These studies will provide more information about apoptotic mechanism caused by *H. pylori*.

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