A study of recombinant protective *H. pylori* antigens

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Abstract

AIM: To construct a recombinant vector which can express *M. 26000* outer membrane protein (OMP) from *Helicobacter pylori* (Hp), and to obtain the vaccine protecting against *Hp* infection and a diagnostic reagent kit quickly detecting *Hp* infection.

METHODS: The gene encoding the structural *M. 26000* outer membrane protein of *Hp* was amplified from *Hp* chromosomal DNA by PCR, and inserted into the prokaryotic expression vector pET32a (+), which was transformed into chromosomal DNA by PCR, and inserted in the prokaryotic outer membrane protein of the Top10 E. coli strain. Recombinant vector was selected, identified and transformed into BL-21(DE3) E. coli strain. The recombinant fusion proteins were expressed. The antigenicity of recombinant protein was studied by ELISA or immunoblotting and immunized Balb/c mice.

RESULTS: The gene of *M. 26000* OMP was amplified to be 594 base pairs, 1.1% of the cloned genes was mutated and 1.51% of amino acid residues was changed, but there was homogeneity between them. The recombinant fusion protein encoded objective polypeptides of 198 amino acid residues, corresponding to calculated molecular masses of *M. 26000*. The level of soluble expression products was about 38.96% of the total cell protein. After purification by Ni-NTA agarose resin columniation, the purity of *r 26000* OMP was amplified to be 99.6%. The recombinant fusion proteins were expressed. The antigenicity of recombinant protein was studied by ELISA or immunoblotting and immunized Balb/c mice.

CONCLUSION: *M. 26000* OMP may be a candidate vaccine preventing *Hp* infection.

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INTRODUCTION

*Helicobacter pylori* (Hp) is a microaerophilic, spiral and gram-negative bacillus first isolated from human gastric antral epithelium in 1982. It is recognized as a human-specific gastric pathogen that colonizes the stomachs of at least half of the world’s population[1]. Most infected individuals are asymptomatic. However, in some subjects, the infection is associated with the development of peptic ulcer, gastric adenocarcinoma, mucosa-associated lymphoid tissue (MALT) lymphoma and primary gastric non-Hodgkin’s lymphoma[2-11]. Furthermore, this organism was recently categorized as a class I carcinoma by the World Health Organization[12], and direct evidence of carcinogenesis was recently demonstrated in an animal model[13,14]. Immunization against the bacterium represents a cost-effective strategy to reduce the incidence of global gastric cancer and would also have a major impact on *H. pylori*-peptic ulcer disease[15]. The selection of antigenic targets is critical in the design of an *Hp* vaccine. To date, this area is scarcely touched upon. The majority of studies focused on the urease enzyme, heat shock protein, VacA, and so on[1,18-19], but not *M. 26000* outer membrane proteins. So, in this study, the recombinant plasmid of *H. pylori* M.26000 outer membrane protein genes was constructed, and expressed for development of *Hp* vaccine.

MATERIALS AND METHODS

Materials

A well-characterized strain, *H. pylori* (*Hp*), was afforded by the Department of Microbiology, Chongqing University of Medical Sciences. Top10, BL21 E. coli strain and pET32a(+) plasmid were presented by the Institute of Viral Hepatitis of Chongqing University of Medical Sciences. Restriction enzymes (HindIII, BamHI) and T, DNA ligase were purchased from Promega, TagDNA polymerase was produced by Immunology Department of the former Beijing Medical University. Isopropyl-β-D-thiogalactopyranoside (IPTG), dNTP and oligonucleotide primers were obtained from Sigma Chemical Co. and so on.

Cloning of *Hp* M.26000 OMP gene

Oligonucleotide primers were designed to amplify *H. pylori* open reading frame (ORFs) of *M. 26000* outer membrane protein based on the published genome sequence[20]. The primers were designed with a BamHI site incorporated into the 5’ end and a Hind III site at the 3’ end as follows(5’-3’): GCGGATCCA TGTTAGTTACAAAA CTTGCC HI site incorporated into the 5’ end and a dIII site at the 3’ end (forward) and AAGCTTAATGGA TTTTCTTT (reverse). Genomic DNA prepared from Chongqing *H. pylori* strains was used as the template in the PCR. The PCR cycle consisted of 30 cycles of denaturation at 94°C for 60s, annealing at 58°C for 45s, with an extension step at 72°C for 90s. Products were visualized on 1.5% agarose gels.

Fifty µL Top10 incubated at 37°C overnight was added into 2mL Luria-Bertani broth and routinely grew at 37°C, and shaken at 300r·min⁻¹ for 4h. When optical density at 600nm was 0.5, it was ultracentrifuged at 10000r·min⁻¹ for 10min at 4°C. The resulting deposits were suspended with 100mmol·L⁻¹ CaCl₂ 150µL and incubated at 0°C for 2h. Ten µL connected products(above) was resuspended and incubated at 0°C for 30min, at 42°C for 2min and at 0°C for 2min respectively. At last, it was incubated at 37°C at 180r·min⁻¹ for 30min after the addition of 1mL LB broth, 200µL was collected and spred onto an LB plate containing 100mg·L⁻¹ ampicillin as the selectable marker and incubated at 37°C overnight.
**Extraction and expression of recombinant plasmid**

The next day, the single cloned bacterial drop was selected, and cultured in 2mL LB broth containing 100mg·L⁻¹ ampicillin at 37°C overnight at 300r·min⁻¹, then recombinant plasmids were extracted and screened with plasmid extraction kit according to the manufacturer's instruction, in the meantime, identified by PCR and restriction enzyme digestion. The recombinant plasmids were selected and transformed into competent BL21(DE3) E.coli strains using standard procedures. BL21 E.coli strains containing recombinant plasmid were grown until mid-log phase (optical density at 600nm=0.5 to 1.0), and expression of the fusion proteins was induced by addition of 0.5-4.0mmol·L⁻¹ IPTG for 4h. Following induction, the bacteria were harvested by ultracentrifugation at 12000r·min⁻¹, resuspended in protein-buffer and seethed for 5min. Total protein was electrophoresed on SDS-PAGE gel and stained with coomassie.

**Immunoblotting analysis**

Briefly, the M₂₆₀₀₀ OMP was purified using Ni-NTA agarose resin after bacteria were cultured and broken down by microwave with the energy of 600W×35% for 40min, ultracentrifuged (10000g, 15min, 4°C), and then quantified. *H. pylori M₂₆₀₀₀ outer membrane protein*-specific antibody was produced following subcutaneous immunization of the New Zealand rabbits, while age-matched control rabbits were immunized with PBS as described previously[17]. Serum antibody specificity was determined by ELISA or immunoblotting following electrophoretic transfer of SDS-PAGE-separated (150g·L⁻¹ acrylamide) H. pylori M₂₆₀₀₀ outer membrane protein to 0.45μm pore size PVDF membrane. After a 30min wash in Tris-saline blotting buffer, antigen-impregnated PVDF strips were incubated with the rabbit sera for 2h at RT. After washing, bound rabbit antibodies were detected by incubation of the strips in alkaline phosphatase-conjugated goat anti-rabbit IgG antibody for 1h at RT.

**Prophylactic immunization**

Six- to eight-week-old mice were immunized three times by subcutaneous immunization using emulsified M₂₆₀₀₀ OMP with Freund’s adjuvant at intervals of 1, 14 and 21 days respectively, to produce antibody responded to M₂₆₀₀₀ outer membrane protein. The dose consisted of 1mL (100mg·L⁻¹) of purified M₂₆₀₀₀ OMP and 1mL complete Freund’s adjuvant. Thereafter, the dose consisted of 0.5mL OMP and 0.5mL incomplete Freund’s adjuvant. Age-matched control mice were immunized with PBS. The antibody titers in immunized mice were monitored by ELISA with purified fusion protein. Mice were challenged with a single dose of 10⁶ *H. pylori* organisms 7 days after the last immunization. Twenty-eight days after challenge, the mice were killed by cervical dislocation. The stomach of each animal was removed, bisected longitudinally, and pinned out. Full-thickness tissue was taken from the antrum-body area of one-half of each stomach and placed into 0.2mL of urease test medium. Urease activity in the sample, identified by a distinctive color change of the stomach was fixed in 100mL·L⁻¹ buffered formalin and embedded in paraffin. Longitudinal sections, stained with a modified May-Grünwald Giemsa stain, were scanned by full length under light microscopy. Mice were considered protected or not according to the previously report[17].

**Statistical analysis**

The Student test was used to evaluate the presence or absence of experimental infection in test and control animals as well as the anti-M₂₆₀₀₀ outer membrane protein response to immunization. *P* values <0.05 were considered as statistically significant.

**RESULTS**

**PCR amplification of H. pylori M₂₆₀₀₀ OMP gene**

According to the literature, the gene encoding the M₂₆₀₀₀ outer membrane protein, was amplified by PCR with Chongqing *H. pylori* strain’s chromosomal DNA as the templates. The cloning products were electrophoresed and visualized on 10g·L⁻¹ agarose gel(Figure 1). It revealed that M₂₆₀₀₀ OMP DNA fragment amplified by PCR contained a gene of approximately 594 nucleotides, which was compatible with the previous reports[17].

**Identification of recombinant plasmid by restriction enzyme digestion**

The recombinant plasmids pET32a(+) were all digested by HindIII or BamHI, and by HindIII and BamHI simultaneously, then digestive products were visualized on 10g·L⁻¹ agarose gel electrophoreses (Figure 2). It demonstrated that recombinant plasmid contained the objective gene.

**Sequence analysis of cloned M₂₆₀₀₀ OMP nucleotide**

The nucleotide sequence of the cloned genes inserted in pET32a(+) was analyzed by automated sequencing across the cloning junction, using the universal primer T₎. The results were: the cloned genes contained 594 nucleotides with a promoter and a start codon coding a putative protein of 198 amino acid residues with a calculated molecular mass of M₂₆₀₀₀. As compared with previously reports, 1.1% of the cloned genes were mutated, and 1.51% amino acid residues were changed. The homogeneity was about 98% between them. The cloned gene and mutative protein sequences were published in GenBank (AY 033499).

**Analysis of the recombinant fusion protein**

Following recombinant vector transformed into BL21 *E. coli* strains,
the fusion protein was amply expressed. Its molecular mass was M, 46000 by 150g/L SDS-PAGE gel analysis (the expression of the pET32a(+) vector, M,20000). After the recombinant bacteria broken down by microwave and ultracentrifuged (10000r·min⁻¹, 15min, 4°C), the level of soluble fusion protein in the supernatant was about 38.96% of total cell protein. After purification by Ni-NTA agarose resin columnation, the purity of objective protein was about 90% (Figure 3).

**Figure 3** 150g/L SDS-PAGE analysis of the fusion protein expressed in BL21(DE3). Lane1: Molecular weight marker; 2Lane: BL21 after 4h induction with IPTG; Lane3-9: BL21/recombinant vector expression after 4h induction with 0.5,1,1.5,2,2.5,3,4mMol/L IPTG respectively; Lane10: BL21/pET32a(+) vector expression after 4h induction with IPTG.

**Antigenicity study of recombinant fusion protein**
Sera were obtained from persons infected and not infected with H. pylori respectively. The recombinant fusion protein was recognized by the H. pylori positive sera, not recognized by the H. pylori negative sera, while the expressed protein of BL21/pET32a(+) not recognized by the H. pylori positive sera; the recombinant fusion protein was also recognized by the rabbit sera immunized with M,26000 OMP, however the expressed protein of BL21/pET32a(+) not recognized by the rabbit sera immunized with M,26000 OMP.

**Prophylactic efficacy with H. pylori M,26000 OMP**
Subcutaneous immunization with H. pylori OMP and FA(Freund’s adjuant) conferred immune protection against H. pylori challenge in 19 (95%) of 20 mice. In contrast, 15 (100%) of 15 naïve control animals were infected with H. pylori. These differences were statistically significant (P<0.05). The protection from infectious challenge was correlated with serum antibody reactivity to M,26000 OMP by immunoblotting. Similar reactivity was absent in the sera collected from same animals prior to immunization, while sera from mice sham immunized with PBS and FA failed to display similar immune responsiveness.

**DISCUSSION**
The outer membrane is a continuous structure on the surface of gram-negative bacteria and an asymmetric bilayer with phospholipids in the inner monolayer and the bulky glycolipid lipopolysaccharide (LPS) in the outer monolayer, in bacterial pathogens, has bilateral particular significance as a potential target for protective immunity and avoiding the host’s immune system. Outer membrane vaccines have been used with considerable success to induce protection against a number of organisms, including H. pylori the heat shock protein, urease A, B and so on. M,26000 OMP is a low molecular mass Hor protein belonging to family I of H. pylori. An earlier study showed that it was commonly expressed in all H. pylori strains examined so far. Furthermore, no cross-reaction is shown when antibodies (polyclonal and monoclonal) to H. pylori low-molecular outer membrane protein are used to immunoscreen closely related species of helicobacter, campylobacter, or a diverse range of other bacteria. Hp low molecular outer membrane protein is unique.

In our study, 1.1% of the cloned genes was mutated. 1.51% of amino acid residues was changed as compared with other reports. The reasons of difference might be summarized as follows: (1) H. pylori chromosomal DNA as templates were different; (2) there is heterogeneity among strains; and (3) H. pylori was provided with the ability of transformation, which could lead to H. pylori varied and genome resited. But there was homogeneity between them. The purified recombinant M,26000 OMP antigen could be recognized by the sera of patients infected with H. pylori and rabbit sera immunized with the recombinant protein. Moreover, in animal model, Balb/c mice immunized with the recombinant fusion protein were protected against H. pylori infection. These were consistent with previous reports.

While being an immunogenic marker, M,26000 OMP showed a high sensitivity and specificity. Moreover, a significant association was found between the serologic response to M,26000 antigen and malignant outcome of H. pylori infection. The serum test for detecting antibody with low molecular weight proteins of H. pylori could be useful for identifying H. pylori-infected patients at risk of peptic ulcer or malignancy. The results showed that M,26000 OMP is not only an immunogenic marker for detecting Helicobacter pylori infection and gastric carcinoma, but also a true vaccine candidate.

In addition to constructing the recombinant vector, we also tried to seek live carriers, because antigen delivery systems can influence the immune response qualitatively as well as quantitatively. Immunization via the mucosal route offers the advantage that it has the potential to stimulate both mucosal immunity and systemic immunity. It is simple, safe and can be used for the immunization of large population groups. Another advantage is the existence of the common mucosal immune system which induced protective immune responses at one mucosal site to be expressed at another. So live carriers on oral route are ideal vaccine delivery systems and are being increasingly used to express large amounts of protective recombinant antigens. We are investigating live carriers to provide a mucosal vaccine vector to deliver M,26000 OMP to antigen-presenting cells on the mucosal surface.

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