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Study of Hgp44 from *Porphyromonas gingivalis* on inducing HUVECs to secrete IL-6 and IL-8

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ABSTRACT. The aim of this study was to clone, express the gene of Hgp44 in adhesin domains of gingipains from *Porphyromonas gingivalis* and purify the protein. Furthermore, the effect of Hgp44 from *P. gingivalis* on inducing HUVECs to secrete IL-6 and IL-8 was evaluated. The Hgp44 gene fragment was amplified by polymerase chain reaction, and then inserted into the cloning vector pMD18-T and linked with a prokaryotic expression vector pET22b to construct the recombinant expression plasmid pET22b-Hgp44. Fusion protein expression was induced by IPTG, and it was purified by immobilized metal-chelating affinity chromatography (IMAC) using an Ni^{2+} matrix column. HUVECs were cultured *in vitro* and different concentrations of Hgp44 were added to confluent HUVEC monolayers and incubated for 2, 8 and 24 h. We extracted the supernatants and then used ELISA kits to test the changes in IL-6 and IL-8 levels. Finally, a 1100-bp fragment

was successfully amplified, and the expression of the fusion protein was examined by SDS-PAGE and Western blot analysis, and the data showed that the protein was 44 kDa in size and expressed mostly in the form of inclusion bodies. The purification of the fusion protein was achieved using Ni^{2+} affinity chromatography. About 3.5 mg/L fusion protein was obtained. Hgp44 could induce HUVECs to secrete IL-6 and IL-8 levels, which were remarkably increased. In a word, Hgp44 was successfully expressed in a prokaryotic expression system and purified by IMAC using the Ni^{2+} matrix column. The effect of Hgp44 in inducing HUVECs to secrete IL-6 and IL-8 was demonstrated.

Key words: *Porphyromonas gingivalis*; Hgp44; Prokaryotic expression; Protein purification; Human umbilical vein endothelial cell (HUVEC); Interleukins 6 and 8

INTRODUCTION

Periodontitis is a chronic infection disease that begins with inflammation in periodontal tissues and finally causes resorption of alveolar bone and subsequent tooth loss. *Porphyromonas gingivalis* is a black-pigmented Gram-negative anaerobic bacterium that is frequently isolated from deep subgingival pockets in chronic periodontal disease and implicated as one of the most important etiological agents of adult periodontitis (Holt et al., 1988). A number of virulence factors have been implicated in the pathogenicity of *P. gingivalis*, such as fimbriae, lipopolysaccharides, various proteases and hemagglutinins (Isogai et al., 1988; Holt and Bramanti, 1991).

Gingipains consist of two types of protease, namely arginine-specific cysteine protease (Arg-gingipain, Rgp) and lysine-specific cysteine protease (Lys-gingipain, Kgp), which are produced by *P. gingivalis* (Pike et al., 1994; Potempa et al., 1995). Molecular genetic analyses have suggested that Rgp is encoded by two separate rgp genes, rgpA and rgpB, and that Kgp is encoded by a single gene, kgp (Nakayama et al., 1995). rgpA and kgp are composed primarily of three parts: a propeptide domain, a catalytic domain, and C-terminal adhesion domain. Furthermore, the adhesion domain consists of four subdomains (Hgp15, Hgp17, Hgp27, and Hgp44) (Kadowaki et al., 2000). Moreover, the adhesion subdomains are highly homologous between rgpA and kgp. In comparison to rgpA, rgpB consists of a propeptide domain and a catalytic domain without adhesion domain. The adhesion domain is also encoded by the hemagglutinin-encoding gene hagA (Han et al., 1996).

Analyses of various Rgp- and/or Kgp-deficient mutants of *P. gingivalis* have revealed that Rgp and Kgp are crucial determinants of *P. gingivalis* virulence (Baba et al., 2002). Especially, the adhesion domain proteins play a central role in the function of gingipains.

Cardiovascular disease (CVD), including coronary heart disease, is the most important clinical manifestation of atherosclerosis. Atherosclerosis is a chronic disease characterized by lipid retention and inflammation (Higashi et al., 2009). Inflammatory processes have become an integral part of the pathophysiology and are presumed to be involved in all stages of atherosclerosis from the initiation and progression to the final stages of infarction (Ross, 1999). A number of epidemiological studies have shown important relationships between periodontal disease and CVD (Mattila et al., 1995; Morrison et al., 1999). Moreover, the presence of *P. gingivalis* in atherosclerotic plaques has been found and studied (Kozarov et al., 2005;

Inomata et al., 2009). Chronic infections are involved in the pathogenesis of CVD through the release of cytokines and pro-inflammatory mediators, which may initiate a cascade of inflammatory reactions causing endothelial damage and cholesterol plaque formation (Ridker et al., 1998), where IL-6 and IL-8 are important inflammatory factors, as they contribute to atherosclerotic plaque development and destabilization. Previous studies have demonstrated that *P. gingivalis* disrupts the ability of endothelial cells to produce IL-1, IL-6 and IL-8 (Brodala et al., 2005). These data suggest that such "chemokine paralysis" suppresses the host's ability to recruit and localize neutrophils to gingival sites of the infection.

Recently, studies have found that Hgp44 adhesion on the bacterial cell surface is essential for *P. gingivalis*-induced platelet aggregation in platelet-rich plasma. In addition, Hgp44 of *P. gingivalis* has been reported to inhibit HIV-1 infection by blocking HIV-1 entry (Xie et al., 2006). Therefore, it is important for us to further investigate the function of Hgp44.

The aim of this study was to clone and express the gene of Hgp44 in adhesin domains of gingipains from *P. gingivalis* and to purify the protein. Furthermore, the effect of Hgp44 from *P. gingivalis* on inducing HUVECs to secrete IL-6 and IL-8 was determined.

MATERIAL AND METHODS

Chemicals and reagents

The pMD18-T vector, T4 DNA ligase, Taq DNA polymerase, DNA molecular mass markers, and all restriction enzymes were purchased from TaKaRa (Dalian, China). The pET22b vector was obtained from Novagen (USA). The Ni-NTA kit was purchased from Qiagen (Germany). The His-Tag monoclonal antibody was obtained from Biovision and the peroxidase-conjugated goat anti-mouse IgG was provided by MultiSciences Biotech Co., Ltd. (Hangzhou, China). IL-6 and IL-8 ELISA kits were from R&D (USA). All other chemical reagents were of analytical grade and provided by Sangon Biotech Co., Ltd. (Shanghai, China).

Bacterial strains and growth

P. gingivalis strain ATCC 33277 was grown in Trypticase soy broth supplemented with 5 μg/mL hemin and 1 μg/mL menadione under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2). *Escherichia coli* strains were grown in Luria-Bertani broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) at 37°C with agitation, supplemented with 100 μg/mL ampicillin in the case of strains harboring pET22b. For over-expression of cloned genes, cultures were grown in NZCYM broth (0.5% yeast extract, 0.1% casamino acid, 1% NZ-Amine A, 0.5% NaCl, and 0.2% $MgSO_4 \cdot 7H_2O$).

Synthesis of the Hgp44 gene by polymerase chain reaction (PCR)

The Hgp44 gene fragment was amplified by PCR from genomic DNA isolated from *P. gingivalis* (ATCC 33277) using the Genomic DNA Isolation kit (BBI, Canada), following manufacturer instructions. The primers were synthesized by Sangon Biotech Co., Ltd. and were as follows: forward (5'-C**CATATG**AGCGGTCAGGCCGAG-3') and reverse (5'-G**CTCGAG**TGCCGTAATCGTCTCTTC-3'), containing the *Nde*I and *Xho*I restriction

sites (bold), respectively. The reaction was carried out according to the following reaction cycles in a Peltier Thermal Cycler: initial denaturation at 95°C for 2 min followed by 35 consecutive cycles of denaturation at 94°C for 1 min, annealing for 1 min at 60°C, and extension at 72°C for 2 min, and then a final extension at 72°C for 10 min. Subsequently, the products were separated on a 1% agarose gel, stained with 0.5 mg/mL ethidium bromide and visualized with a UV transilluminator. Finally, the amplified products were excised using the DNA Gel Extraction kit (Axygen, USA).

Construction of the recombinant plasmid pET22b-Hgp44

The purified products were cloned into the pMD18-T cloning vector (TaKaRa) according to the manufacturer protocol, and the recombinant plasmids were then transformed into competent *E. coli DH5α* cells and confirmed by restriction enzyme digestion and sequencing. The correct recombinant cloning vector was named pMD18T-Hgp44.

A positive recombinant cloning plasmid that contained the Hgp44 gene was digested with *Nde*I and *Xho*I, and the Hgp44 gene was sub-cloned into the corresponding region of pET22b vector to construct the recombinant expression plasmid pET22b-Hgp44 and transformed into competent *E. coli BL21* (*DE3*) cells for inducible expression.

Expression of the fusion protein

The confirmed recombinant plasmids containing the fusion gene pET22b-Hgp44 were transformed into competent *E. coli BL21* (*DE3*) cells. We optimized the conditions for induction to obtain as much recombinant protein as possible. Different concentrations of isopropyl-β-D-thiogalactoside (IPTG; 0.1, 0.3, 0.5, 0.7, 0.9, 1.2, and 1.5 mM) and different induction times (1, 2, 3, 4, 5, 6, and 7 h) were used. The recombinant protein was expressed on a satisfactory scale as follows. Freshly transformed cells were grown in NZCYM medium containing 100 μg/mL ampicillin at 37°C with aeration (220 rpm). Overnight cultures were diluted 1:100 into fresh NZCYM medium. When cells were grown to an optical density of 0.6 at 600 nm, protein expression was induced by the addition of IPTG to a final concentration of 0.5 mM at 37°C for 6 h.

A 50-mL portion of induced culture was harvested by centrifugation at 4000 *g* for 30 min and the pellets stored frozen at -20°C. The cell pellets were thawed on ice and resuspended in 5 mL lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). The cells were then lysed by sonication (6X 10 s with 10-s pauses at 200-300 W) in an ice bath to reduce viscosity, and the lysate was centrifuged at 10,000 *g* for 30 min at 4°C. The clear supernatant (soluble fraction) was collected and the remaining pellet (insoluble fraction) containing inclusion bodies was resuspended in an equal volume of lysis buffer. All fractions of the extraction procedure were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970) and stained with 0.1% Coomassie Brilliant Blue R-250.

Western blot analysis of the recombinant protein

For immunoblotting detection of the Hgp44 linked to six histidine residues, protein samples in loading buffer (0.1 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.2 M β-mercaptoethanol, and 0.2% bromophenol blue) were boiled for 5 min. After centrifugation

for 1 min, the samples were subjected to 10% SDS-PAGE (80 V for 5% gel and 120 V for 10% gel), and then electroblotted onto a 0.45-μm pore size nitrocellulose membrane (Millipore) at a constant voltage of 60 V in an ice bath for 90 min using a Mini-Trans blot cell (Bio-Rad) with the transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol). Subsequently, nonspecific binding sites were blocked by incubating the membranes with 5% (w/v) skim milk in TBST (100 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.1% Tween 20) for 2 h at room temperature with agitation. The membrane was then incubated with primary antibodies (His-Tag Monoclonal Antibody at 1:2000 dilution in TBST) overnight at 4°C. After washing three times with TBST, goat anti-mouse IgG conjugated with HRP was added and the membrane incubated for 2 h at room temperature. The membrane was washed three times with TBST and visualized with VersaDoc Imaging System (BIO-RAD).

Purification of the recombinant protein

A 50-mL portion of induced culture was harvested by centrifugation (4000 *g*, 30 min) and stored at -20°C until required. The cell pellets were thawed on ice and resuspended in buffer B (8 M urea, 10 mM Tris-HCl, 100 mM NaH_2PO_4, pH 8.0) at 5 mL per gram wet weight. The cells were incubated with agitation for 30 min at room temperature and the lysate centrifuged at 12,000 *g* for 30 min at room temperature to pellet the cellular debris. Subsequently, the supernatant containing the fusion protein was loaded onto an Ni-NTA column equilibrated with buffer B to purify the Hgp44 protein, following manufacturer instructions. The column was washed with buffer C (8 M urea, 10 mM Tris-HCl, 100 mM NaH_2PO_4, pH 6.3) and eluted with buffer E (8 M urea, 10 mM Tris-HCl, 100 mM NaH_2PO_4, pH 4.5). The eluate was collected and analyzed by SDS-PAGE and Western blot. At the end, the protein concentration was determined using a BCA protein assay kit.

Cell culture and stimulation experiments

HUVECs were purchased from Cell Bank, Chinese Academy of Sciences. Cells were cultured according to manufacturer specifications using as endothelial cell growth medium, DMEM (Gibco, USA), supplemented with penicillin/streptomycin (50 mg/L) and 10% fetal bovine serum at 37°C in 5% CO_2 in oxygen tissue culture flasks. The growth medium was changed every other day until a confluence of 80-90% was reached for stimulation experiments.

Three different concentrations of Hgp44-DMEM were prepared (10, 100 and 1000 μg/mL), and DMEM served as a control. Confluent cells were used in all experiments, where HUVECs were seeded on 12-well plates and grown to confluency. Plates were washed three times with phosphate-buffered saline, and then 1 mL of different concentrations of Hgp44-DMEM and DMEM was added to each well. The cells were incubated for 2, 8 and 24 h at 37°C, in a humidified and 5% CO_2 atmosphere.

Measurement of IL-6, IL-8 by ELISA

After Hgp44-treated HUVECs were incubated under the conditions indicated, the culture supernatants were collected and stocked at -80°C until use. The amount of IL-6 and IL-8 was measured using ELISA kits (R&D), according to manufacturer instructions. Means and

standard deviations (SD) were calculated for three separate experiments. Experiments were performed in triplicate and the data were calculated using the CurveExpert software.

Statistical analysis

The data obtained were reported as means ± SD. Statistical significant differences between samples were evaluated by the *t*-test, which was carried out with the SPSS 16.0 software on a personal computer. $P \leq 0.05$ was considered to be significant.

RESULTS

Construction of the recombinant plasmid pET22b-Hgp44

The nucleotide sequences encoding the Hgp44 gene were amplified from *P. gingivalis* (ATCC 33277) by PCR using specifically designed primers, which contained the *Nde*I and *Xho*I sites, and verified by agarose gel electrophoresis and sequencing. The size of the resulting DNA fragment was about 1100 bp on an agarose gel (Figure 1), which showed 99% similarity with the corresponding region of rgpA.

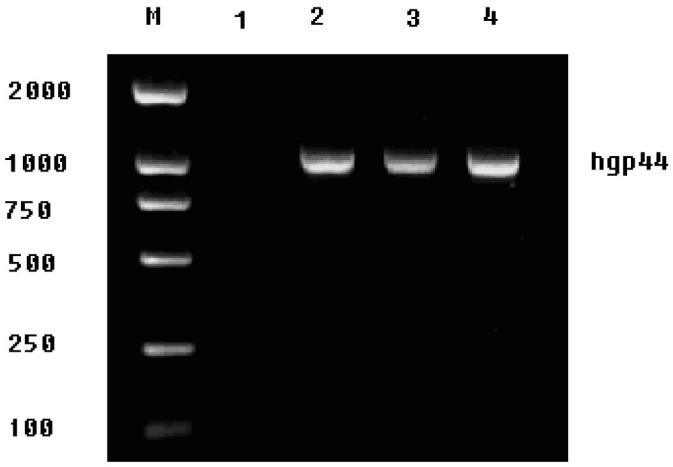

Figure 1. PCR products of target genes. *Lane M* = DNA marker; *lane 1* = negative control; *lanes 2-4* = PCR fragments of Hgp44.

Expression of the recombinant protein

E. coli BL21 (*DE3*) cells were transformed with the confirmed recombinant expression plasmids to obtain the engineered bacteria. Optimal expression conditions were selected as described in Materials and Methods to obtain maximal amounts of the recombinant protein. It was shown that optimal induction conditions were established with the addition of 0.5 mM IPTG for 6 h at 37°C (Figure 2), and the data showed that the effect of different concentrations of IPTG was not notable. Subsequently, we examined the solubility of the expressed

recombinant protein by analyzing the supernatant and pellet of the cell lysate, and found that the recombinant protein was distributed in inclusion bodies in an insoluble form (Figure 3).

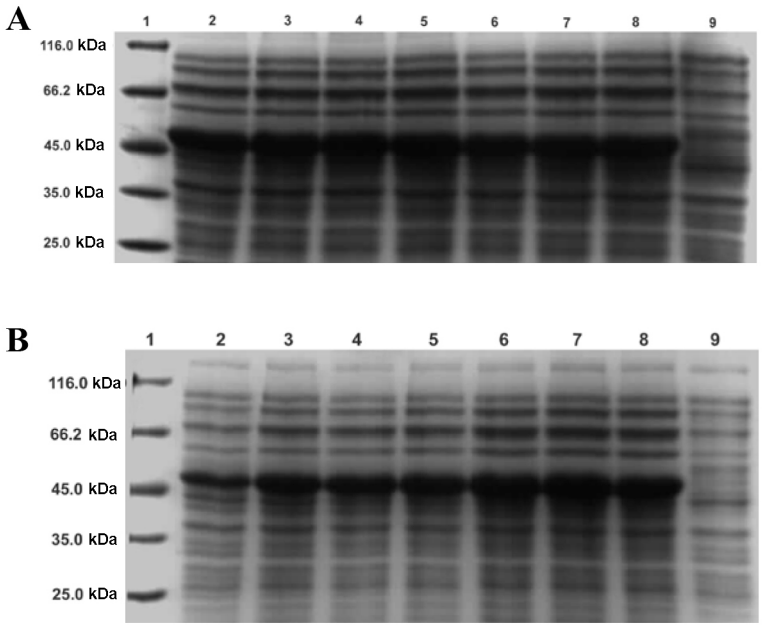

Figure 2. Optimization of expression conditions by the 10% gel electrophoresis of total cellular protein. **A.** Expression of recombinant protein at different concentrations of IPTG for 5 h. *Lane 1* = molecular weight marker; *lane 9* = without IPTG induction; *lanes 2-8* = induction of 0.1, 0.3, 0.5, 0.7, 0.9, 1.2, and 1.5 mM IPTG, respectively. **B.** Expression of recombinant protein at different induction time points. *Lane 1* = molecular weight marker; *lane 9* = induction for 7 h without IPTG treatment; *lanes 2-8* = induction with 0.1 mM IPTG for 1, 2, 3, 4, 5, 6, and 7 h, respectively.

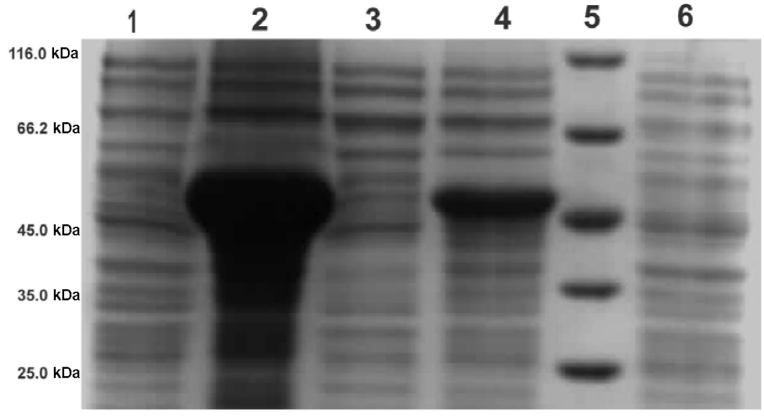

Figure 3. Solubility analysis of the recombinant Hgp44 protein. *Lane 1* = total cellular protein from *Escherichia coli*/pET22b-Hgp44 without induction; *lane 2* = pellet of lysate from induced *E. coli*/pET22b-Hgp44; *lane 3* = supernatant of lysate from induced *E. coli*/pET22b-Hgp44; *lane 4* = total cellular protein from induced *E. coli*/pET22b-Hgp44; *lane 5* = molecular weight marker; *lane 6* = total cellular protein from *E. coli*/pET22b without induction.

Purification and identification of the recombinant protein

Immobilized metal-chelating affinity chromatography with an Ni^{2+} matrix column was used to isolate and purify the recombinant protein containing the His6-tag. The purified recombinant protein Hgp44 was recognized by SDS-PAGE and Western blot with anti-His monoclonal antibody (Figure 4). The molecular weight of the purified protein was estimated by SDS-PAGE to be approximately 46 kDa including the artificial histidine tag of about 2 kDa, so it agreed with the theoretical molecular weight of 44 kDa. Based on spectrophotometric measurement of protein concentration in the eluted fraction, it was calculated that about 3.5 mg purified recombinant protein Hgp44 was obtained per liter of bacterial culture.

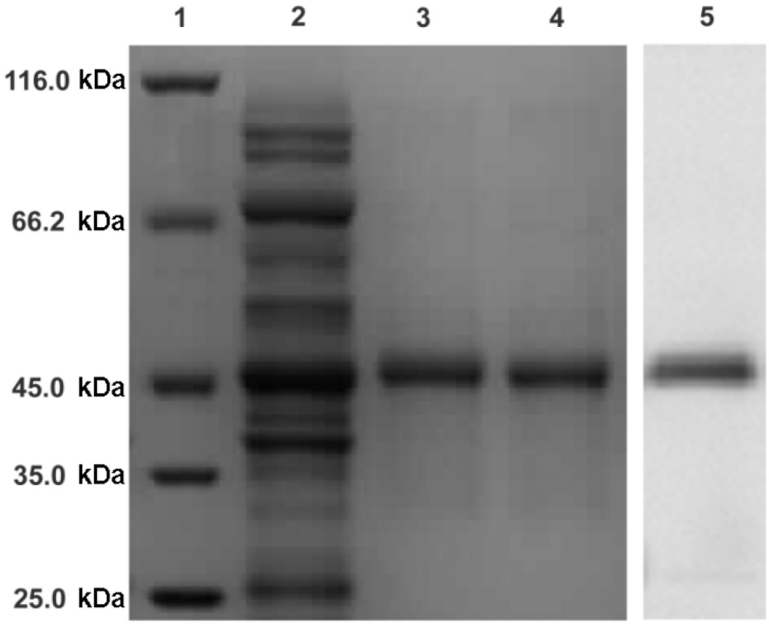

Figure 4. Purification of the recombinant Hgp44 protein and Western blot analysis. *Lane 1* = molecular weight marker; *lane 2* = crude lysates; *lanes 3* and *4* = the purified recombinant Hgp44 protein; *lane 5* = Western blot analysis of purified protein using an anti-His tag monoclonal antibody.

Hgp44 on inducing HUVECs to secrete IL-6 and IL-8

HUVECs were stimulated for 2, 8 and 24 h with three different concentrations of Hgp44 (10, 100 and 1000 µg/mL). As shown in Figure 5, we found that IL-6 production increased with time and concentration of Hgp44 within a certain range. The production of IL-6 was significantly higher after stimulation for 24 h compared to stimulation for 2 and 8 h ($P < 0.05$). Also, we can see that IL-6 production was very low, when it was stimulated with 1000 µg/mL Hgp44. IL-8 production was the same as IL-6 (Figure 6).

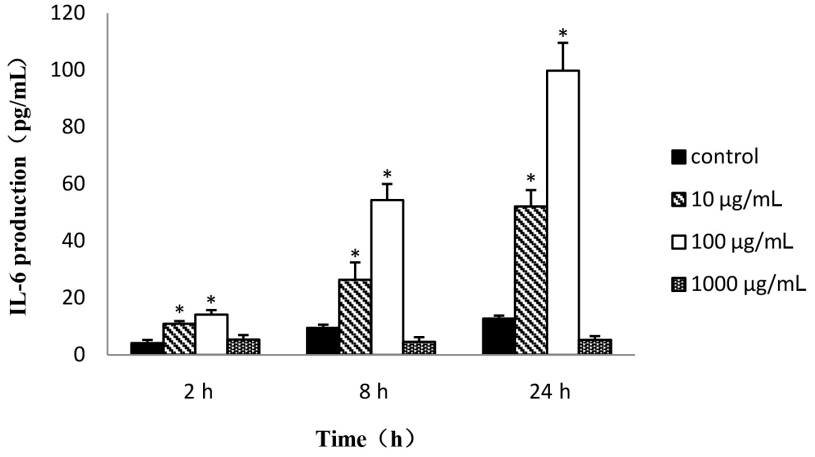

Figure 5. Effects of Hgp44 on inducing HUVECs to secrete IL-6. Three different concentrations of Hgp44 were added to HUVECs, and incubated for 2, 8 and 24 h at 37°C, humidified and 5% CO_2 atmosphere, then culture supernatants were collected for IL-6 ELISA assay as described in the Material and Methods section. Control cultures were incubated with culture media only. Data are reported as means ± SD from three independent experiments. *P < 0.05 compared with control cultures (ANOVA).

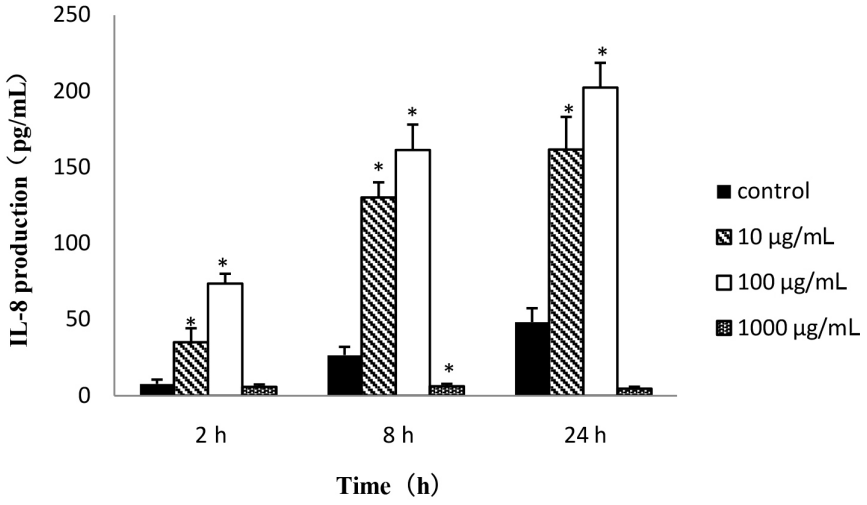

Figure 6. Effects of Hgp44 on inducing HUVECs to secrete IL-8. Three different concentrations of Hgp44 were added to HUVECs, and incubated for 2, 8 and 24 h at 37°C, humidified and 5% CO_2 atmosphere, then culture supernatants were collected for IL-8 ELISA assay as described in the Material and Methods section. Control cultures were incubated with culture media only. Data are reported as means ± SD from three independent experiments. *P < 0.05 compared with control cultures (ANOVA).

DISCUSSION

A number of studies have been conducted on *P. gingivalis*-mediated hemagglutination since it was discovered. Now, researchers have begun focusing on the gingipains and have

made great progress, especially regarding the adhesion domains (Sakai et al., 2007; Ito et al., 2010); among them, Hgp44 is particularly significant. However, there is still a serious challenge in the production of the Hgp44 protein. In this study, we sought a production method to synthesize Hgp44 protein in the prokaryotic expression system.

In the design of recombinant expression systems, a number of significant elements should be considered (Jonasson et al., 2002), so we selected the pET22b vector as an expression vector. The pET22b vector carries an N-terminal pelB signal sequence for potential periplasmic localization and C-terminal His-Tag sequence, which is a pBR322-based plasmid and consists of the T7 promoter, the lac operator and lac repressor, which is encoded by the lacIq gene, and simultaneously, pET22b confers ampicillin resistance to the host (Studier and Moffatt, 1986; Studier et al., 1990). The most ordinary inducer of expression systems is IPTG (Hannig and Makrides, 1998). The lac repressor could bind to the lac operator to repress transcription, in the absence of the inducer IPTG. The results demonstrated that it was well used. At the same time, an *E. coli*-based system was used, which has considerable advantages, such as low production costs, simple cell culture procedures and high yield.

In our current study, the prokaryotic expression system was successfully constructed and produced a recombinant protein in the form of inclusion bodies that corresponded with our expectation. Inclusion bodies are generally recognized as a stress response when recombinant protein is expressed at high rates, which consist of a set of structurally complex aggregates (van den Berg et al., 1999). So far, there is little known about the formation mechanism of inclusion bodies (Villaverde and Carrio, 2003). However, it is recognized that the formation of inclusion bodies is the result of an imbalance between *in vivo* protein aggregation and solubilization, in the expression system (Studier and Moffatt, 1986).

There are many advantages of the recombinant protein in the form of inclusion bodies: to avoid proteolysis, to protect the host cell from product toxicity and to facilitate purification (Barrell et al., 2004). Although the biological activity of protein is not perfect, it could be solubilized using detergents such as urea or guanidine hydrochloride and refolded through various means to acquire the biological activity (Middelberg, 2002; Sorensen et al., 2003). The presence of the fusion of a C-terminal hexa-His tag not only provided an easy means for the purification of the Hgp44 protein by affinity chromatography, but was also demonstrated to impair its biological activity.

P. gingivalis is a major etiological agent in the pathogenesis of adult periodontitis in humans, which can invade endothelial cells via the bloodstream and is found in atheromatous plaque. *P. gingivalis* possesses a great deal of virulence factors, all of which stimulate host cells to release inflammatory mediators and promote infectious disease. Many lines of research have found that *P. gingivalis* play an important role in atheromatous plaque formation. Moreover, *P. gingivalis* infection significantly increases HUVEC expression of VCAM-1, ICAM-1 and E-selectin, and enhanced the production of IL-6, IL-8 and MCP-1 (Roth et al., 2007). IL-6 and IL-8 are pro-inflammatory cytokines that elicit a wide variety of biological activities in various cell types (Kishimoto, 2005). Increased levels of IL-6 and IL-8 correlate with prognosis in patients with the risk of coronary heart disease (Hartford et al., 2006). Furthermore, several *in vitro* experiments have shown that bacterial products of *P. gingivalis*, such as fimbriae, LPS and gingipains, promote IL-6 and IL-8 production in gingival fibroblasts and epithelial cells (Matsushita et al., 2006; Takahashi et al., 2006; Roth et al., 2007).

In the present study, we examined the effects of Hgp44 on inflammatory responses, especially cytokine regulation targeting IL-6 and IL-8 in endothelial cells. Interestingly, as

shown in Figures 5 and 6, we demonstrated that IL-6 and IL-8 secretion was dramatically enhanced in HUVEC by Hgp44 stimulation. These findings indicate the critical roles of Hgp44 in the virulence of *P. gingivalis*.

In conclusion, in this study we successfully constructed an efficient system for the expression and purification of Hgp44 in *E. coli* and obtained adequate amounts of the recombinant protein. We demonstrated the ability of Hgp44 to induce HUVECs to secrete IL-6 and IL-8, and lay a foundation for the study of the interaction between Hgp44 and human endothelial cells, and furthermore for the molecular mechanism of *P. gingivalis* and coronary heart disease.

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