

Full-length article

Effect of *Porphyromonas gingivalis* PrtC on cytokine expression in ECV304 endothelial cells and its level in subgingival plaques from patients with chronic periodontitis¹

Yan-min WU², Li-li CHEN^{2,6}, Jie YAN^{3,6}, Chun-yan ZHUANG⁴, Zhi-yuan GU⁵

²Department of Stomatology, the Second Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310009, China; ³Department of Medical Microbiology and Parasitology, College of Medicine, Zhejiang University, Hangzhou 310006, China; ⁴Department of Stomatology, the Affiliated Hospital of the School of Medicine, Ningbo University, Ningbo 315020, China; ⁵Department of Oral and Maxillofacial Surgery, the Affiliated Hospital of Stomatology, College of Medicine, Zhejiang University, Hangzhou 310006, China

Key words

Porphyromonas gingivalis; collagenase; cloning; cytokine; ELISA

¹ Project supported by a grant from the National Natural Science Foundation of China (No. 30471888).

⁶ Correspondence to Prof Li-li CHEN and Prof Jie YAN.

Phn 86-571-8778-4576.

Fax 86-571-8702-2776.

E-mail yanchen_5657@163.com (Li-li CHEN)

Phn/Fax 86-571-8820-8294.

E-mail Med_bp@zju.edu.cn (Jie YAN)

Received 2006-12-04

Accepted 2007-02-13

doi: 10.1111/j.1745-7254.2007.00599.x

Abstract

Aim: To investigate the effect of the collagenase gene (*prtC*) product of *Porphyromonas gingivalis* on inducing host cells to secrete inflammatory cytokines, and to discuss the correlation between the PrtC level in subgingival plaque samples and clinical parameters. **Methods:** A prokaryotic expression system pET32a-*prtC*-*Escheria coli* BL21DE3 was constructed. Antigenicity and immunoreactivity of the recombinant PrtC protein (rPrtC) was identified by Western blotting. ELISA was applied to detect interleukin (IL)-1 α , IL-8, and TNF- α levels in supernatants from rPrtC-induced human umbilical vein endothelial cells (HUVEC) originated ECV304 cells. Clinical parameters recorded at baseline and after treatment included bleeding on probing (BOP), probing depth (PD), and attachment loss (AL). ELISA was established to measure the PrtC level in 196 subgingival plaque samples from 49 patients with chronic periodontitis. **Results:** After co-cubation with 1 μ g/mL rPrtC for 24 h and with 5 or 10 μ g/mL rPrtC for 12 h, the levels of IL-1 α , IL-8, and TNF- α secreted by the ECV304 cells increased significantly ($P < 0.05$). The PrtC level in the BOP-positive or the ≥ 5 mm AL or > 6 mm PD sites was higher than that in the BOP-negative or the ≤ 2 mm AL or ≤ 6 mm PD sites ($P < 0.05$), respectively. Compared with baseline, the PrtC levels in different AL sites or in the ≤ 6 mm PD pockets decreased remarkably after treatment ($P < 0.01$), but in the BOP-positive or in the > 6 mm PD sites, the PrtC levels changed insignificantly ($P > 0.05$). **Conclusion:** rPrtC is able to directly induce host cells to synthesize and secrete IL-1 α , IL-8, and TNF- α . The PrtC level in subgingival samples is correlated with BOP, AL, and PD.

Introduction

A significant percentage of the human population suffers from periodontitis, a chronic inflammatory disease characterized by the breakdown of periodontal tissue^[1]. The incidence and rate of progression of this disease involve complex interactions among periodontopathic bacteria as well as between periodontopathic bacteria and host cells^[2,3]. These interactions lead host cells to release a broad array of inflammatory cytokines, chemokines, and mediators, some

of which result in the destruction of periodontal supporting tissue, namely alveolar bone, periodontal ligament, and cementum around teeth^[2,3]. The Gram-negative anaerobic bacterium *Porphyromonas gingivalis* is implicated as one of the important etiological agents of chronic and aggressive forms of periodontitis^[4-7]. This organism expresses a number of potential virulence factors, including fimbriae, superoxide dismutase, hemagglutinin, the arginine-X-specific cysteine proteases, and the lysine-X-specific proteases^[8]. In addition, collagenolytic activity of *P. gingivalis* has been

described and is associated with the production of several proteases^[8]. Since collagen is an important component of the periodontium, collagenase activity plays a critical role in tissue destruction and progression of periodontitis^[9]. Specific cleavage of type I collagen has been attributed to the function of the *prtC* gene product, which is referred to as collagenase^[8]. Takahashi and Kato were the first to report the cloning and expression of the *prtC* gene from *P gingivalis* ATCC 33277^[10,11]. While the *prtC* gene product did not exhibit structural similarity with eukaryotic collagenases, it was able to degrade soluble and reconstituted fibrillar type I collagen, heat-denatured type I collagen, and azocoll, but did not degrade synthetic collagenase substrates and did not contain a partial zinc-binding region that is consensus in these enzymes^[12]. These features may reflect the unique nature of this collagenase or perhaps question its status as a true collagenase^[12]. Nonetheless, the inability of a collagenolytic enzyme to degrade native fibrillar collagen does not preclude its involvement in the pathogenesis of periodontal disease^[12]. Proteinase produced by some bacteria could have a direct effect on inducing inflammation besides its specific enzyme activity^[13] and may work in combination with true bacterial or host collagenases^[14,15]. Recent findings indicate that *P gingivalis* induces host collagen degradation by affecting expression, activation, and inhibition of matrix metalloproteinases (MMP) produced by host cells^[8,16]. MMP is thought to be transcriptionally upregulated by pro-inflammatory mediators, such as interleukin (IL)-1 and TNF- α , as well as post-translationally activated by proteases from *P gingivalis*^[16]. However, studies on the *prtC* gene product of *P gingivalis* mainly concentrate on its collagenolytic activity. Its ability to induce host cells to produce inflammatory cytokines has yet not been examined, which is of great importance in understanding its role in the pathogenesis and development of periodontitis.

Several studies have demonstrated that *P gingivalis* is able to invade and activate different cell types in the surrounding tissue of teeth, including endothelial, gingival epithelial cells, as well as periodontal ligament cells^[14,15,17]. Endothelial cells, therefore, can act as primary target cells during infection with *P gingivalis*. Endothelial cells are key players during inflammatory reactions and are able to produce an array of pro-inflammatory mediators, including cytokines such as IL-1, IL-6, IL-8, TNF- α , and lipid mediators like prostaglandins or platelet-activated factor^[17,18]. A previous report has indicated that *P gingivalis* can infect human umbilical vein endothelial cells (HUVEC) and trigger a cascade of events that could lead to endothelial damage, as well as local and systemic inflammation^[17]. Moreover,

host cells sense live *P gingivalis* and its components such as lipopolysaccharides (LPS) or fimbriae differently, which translate into the expression of different inflammatory cytokine profiles^[19]. The objective of the present work is to investigate the effect of the *P gingivalis* PrtC protein on the expression of cytokines in HUVEC-originated ECV304 cells, which has been shown to be a suitable *in vitro* model for the study of endothelial cells^[20,21,22], and subsequently, to determine the possible association of *P gingivalis* PrtC levels with periodontal conditions.

Materials and methods

In vitro study

***P gingivalis* strains and growth condition** The *P gingivalis* strain ATCC 33277 was supplied by the Department of Medical Microbiology and Parasitology, College of Medicine, Zhejiang University, China. They were inoculated on trypticase soy agar supplemented with 5 μ g/mL haemin, 1 μ g/mL vitamin K1, 5% (v/v) sheep blood, and 1 μ g/mL menadione. The plates were incubated in an anaerobic chamber under atmosphere condition of 80% N₂, 10% CO₂, and 10% H₂ at 37 °C for 7 d.

Cloning, sequencing and construction of recombinant expression vector The *P gingivalis prtC* was amplified from type strain ATCC 33277 by PCR with a forward primer containing the site of *Bam*HI: 5'-GGGGGATCCCTCATGCGCTCCGTCATC-3', and a reverse primer with the site of *Xho*I: 5'-GGGCTCGAGTTATTCTTCTCTTTTGTC-3', according to published sequence (GenBank Accession No AB006973). The total volume per reaction was 100 μ L, containing 0.25 mmol/L each dNTP, 1 μ mol/L each of the primers, 3.0 U *Taq-Pfu* polymerase, 20 mmol/L MgCl₂, 100 ng DNA template, and 1 \times PCR buffer (pH 8.3). The PCR conditions were as follows: 94 °C for 5 min, 1 cycle; 94 °C for 30 s, 50 °C for 30 s, 72 °C for 90 s, 10 cycles; 94 °C for 30 s, 50 °C for 30 s, 72 °C for 100 s, 20 cycles; and 72 °C for 10 min, 1 cycle. The PCR products were analyzed by 1.5% agarose gel prestained with ethidium bromide and visualized under UV light. The expected size of the target amplification fragment for *prtC* was 1005 bp.

The *prtC* fragment was cloned into the pUCm-T vector by using the T-A cloning kit according to the manufacturer's instructions (Biocolor, Shanghai, China). The recombinant plasmid (pUCm-T-*prtC*) was first transformed into the *Escherichia coli* strain DH5 α . After nucleotide sequencing confirmed the inserted fragments, the *E coli* DH5 α strains containing pUCm-T-*prtC* or the prokaryotic expression vector pET32a were amplified and then the plasmids were

extracted. pUCm-T-*prtC* and the pET32a vector were both digested with *Bam*HI and *Xho*I. The target fragments of the *prtC* gene and pET32a were recovered and then ligated with a ligation kit (TaKaRa Biomedicals, Otsu, Shiga, Japan). The recombinant expression vector pET32a-*prtC* was transformed into *E coli* strain BL21 (DE3). Then the plasmids were extracted and sequenced again.

Expression and identification of the target recombinant protein The constructed prokaryotic expression system pET32a-*prtC-E coli* BL21DE3 was rotationally cultured in Luria-Bertani (LB) medium at 30 °C under inducement of 0.5 mmol/L isopropyl-1-thio- β -galactoside (IPTG). The supernatant and precipitate were isolated by centrifugation after the bacterium was ultrasonically broken. SDS-PAGE (10%) was used to examine molecular weight, output, and location of the target recombinant protein (rPrnC). rPrnC was then collected and purified by affinity chromatography on a Ni-NTA column (QIAGEN, Hilden, Germany). Following elution with imidazole, contaminated LPS was removed by Triton X-114 extraction. Then the protein was chromatographed on Sephadex G25 columns (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and eluted in phosphate-buffered solution. Solution of the recombinant protein 10 μ g/mL was found to be free of endotoxins by the *Limulus* amoebocyte lysate assay (Bio Whittaker, Walkersville, MD, USA). Rabbits were routinely immunized with 1 mg purified rPrnC or 1 mg whole cells of *P gingivalis* supplemented with complete Freund's adjuvant, administered subcutaneously 4 times. Slide agglutination reaction and ELISA were used to detect the immunoreactivity of rabbit anti-rPrnC antiserum against *P gingivalis* ATCC 33277, *Prevotella melaninogenica* ATCC 25845, *Fusobacterium nucleatum* ATCC 25586, *E coli* ATCC 25922, *Actinomycetemcomitans actinobacillus* Y3, *Veillonella parvula* 990116, and 100 μ g/mL of their sonicated supernatants, respectively. After electrophoresis on 10% SDS-PAGE, the rPrnC protein was then transferred to a polyvinylidene fluoride (PVDF) membrane using the Trans-Blot SD apparatus (Bio-Rad, Richmond, CA, USA). The blot was blocked for 1 h in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% (w/v) skim milk and then incubated with the rabbit antisera (1:1500 dilution) for 1 h. After washing unbound primary antibodies with TBST 3 times for 10 min each, the blot was treated with horseradish peroxidase (HRP)-conjugated sheep anti-rabbit immunoglobulin G (1:3000 dilution; Jackson ImmunoResearch, West Grove, PA, USA) and developed with the ECL system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

ELISA detection of inflammatory cytokine secretion in ECV304 cells induced by rPrnC HUVEC ECV304 was

maintained at a density of 5×10^3 cells/well in a 96-well plate in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified atmosphere of 5% CO₂ in air at 37 °C for 24 h. Then the ECV304 cells were cultured in RPMI-1640 medium supplemented with 2% FBS which contained 1, 5, and 10 μ g/mL rPrnC for 12, 24, or 48 h, respectively. Each concentration and time interval was repeated 3 times. The supernatants were collected for the ELISA detection of IL-1 α , IL-8, and TNF- α (R&D Systems, Minneapolis, MN, USA). Recombinant TNF- α (0.1 μ g, SibEnzyme, Novosibirsk, Siberia, Russia) and RPMI-1640 medium containing 2% FBS were used as positive and negative controls, respectively.

In vivo study

Patients The patients were 49 Chinese chronic periodontitis (CP) patients [24 males aged 29–66 years (mean age, 45.7 \pm 8.9 years) and 25 females aged 33–67 years (mean age, 44.3 \pm 7.6 years)], and 25 individuals with healthy periodontium [11 males aged 26–64 years (mean age, 41.8 \pm 5.8 years) and 14 females aged 22–63 years (mean age, 41.3 \pm 6.8 years)] who were referred to the dental clinic in the Second Affiliated Hospital, College of Medicine, Zhejiang University for dental or periodontal treatment or health monitoring. All the patients were non-smokers without any systemic disease, and had at least 14 teeth. Those who had received professional cleaning or had history of antibiotic therapy during the preceding 3 months were excluded. All of the patients and the healthy individuals underwent a full mouth examination. The criteria of diagnosis for chronic periodontitis were based on the Classification of the Periodontal Diseases issued by American Academy of Periodontology in 1999^[23]. Briefly, the 49 generalized CP patients had >30% sites showing periodontal probing depth \geq 3 mm, clinical attachment loss >1 mm, and radiographic evidence of alveolar bone loss. Individuals with periodontal probing depth less than 3 mm without any clinical attachment loss or radiographic evidence of bone loss and without inflammation of gingivae were considered healthy. All the patients received detailed information concerning the nature of the study and the procedures involved, and their informed consent was obtained. The ethical committee of the College of Medicine at Zhejiang University approved the study protocol.

Collection of samples For each patient, 4 subgingival plaque samples were taken from the bottom of the 4 deepest periodontal pockets of the dentition, preferably 1 pocket from each quadrant, with separate sterile Gracy curettes after supragingival plaque was gently removed. After initial periodontal treatment that mainly included oral hygiene instruction, full mouth supragingival and subgingival scal-

ing and root planning, subgingival plaque samples from the same sites were collected again 4 months later. For individuals with healthy periodontium, samples from the bottom of gingival sulcular were taken with the same method. Each of the samples was placed in 200 μ L lysis buffer, which consisted of 1 mmol/L EDTA, 1% Triton X-100, and 10 mmol/L Tris-HCl (pH 8.0), and stored at -20°C until use. Clinical parameters, such as probing depth (PD), attachment loss (AL), and bleeding on probing (BOP) at 6 sites of each tooth of the full dentition were recorded at baseline and after initial periodontal treatment. The 6 sites were the mesial-buccal, middle-buccal, distal-buccal, mesial-lingual/palatal, middle-lingual/palatal, and distal-lingual/palatal sites of each tooth.

PCR detection of *P. gingivalis* in subgingival plaque samples Lysis buffer 100 μ L containing subgingival plaque samples was boiled for 10 min, and 10 μ L supernatant was directly used as a template in the PCR. A multiplex PCR assay was established to detect the *P. gingivalis* *16SrDNA* and *prtC* genes. The sequences of the primers specific for the *P. gingivalis* *16SrDNA* gene were: 5'-AGG CAG CTT GCC ATA CTG CG-3' (sense) and 5'-ACT GTT AGC AAC TAC CGA TGT-3' (antisense)^[24]. For the *prtC* gene they were: 5'-ACA ATC CAC GAG ACC ATC-3' (sense) and 5'-TTC AGC CAC ACC GAG ACG-3' (antisense)^[25]. PCR amplification was performed in a total volume of 100 μ L, containing 10 μ L of the template, 10 μ L PCR buffer (pH 8.3), and 3U EX-*Taq* polymerase (TaKaRa Biomedicals, Japan), 0.25 mmol/L dNTP, 2.5 mmol/L MgCl_2 , 250 nmol/L primers each for *P. gingivalis* *16SrDNA* and *prtC* genes. The PCR programs for the detection of *P. gingivalis* included an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 52°C for 30 s, extension at 72°C for 1 min, and then a final extension step at 72°C for 7 min. In each of the PCR assays, 10 ng *P. gingivalis* ATCC 33277 DNA preparation was co-amplified with the subgingival plaque samples as the positive control for the detection of *P. gingivalis*. The expected sizes of the PCR products amplified from the *P. gingivalis* *16SrDNA* and *prtC* genes was 404 bp and 584 bp^[24,25]. Each reaction product 10 μ L were mixed with 10 μ L of 2 \times loading buffer and fractionated in a 2% agarose gel containing ethidium bromide (1 $\mu\text{g}/\text{mL}$), using a 100 bp DNA ladder (Promega, Madison, WI, USA) as a size marker, and visualized under UV light.

ELISA detection of PrtC levels in subgingival plaque samples The subgingival plaque samples were sonicated, and the protein concentration in each of the samples was measured by UV spectrophotometry. Each sample was diluted to 20 $\mu\text{g}/\text{mL}$ protein with coating buffer in ELISA. By using 0.1 mL of the diluted samples as the coating antigen,

the self-prepared rabbit anti-rPrtC serum (1:1000 dilution) as the first antibody, and HRP-labeled sheep anti-rabbit IgG (1:3000 dilution) as the second antibody, the PrtC levels in the samples were detected. A model 680 microplate reader (Bio-Rad, USA) was used to detect the A_{490} value. The ELISA result of a sample was considered positive if its A_{490} value was over the mean plus 3 times that of standard deviation (SD) of the sonicated sulcular samples with the same protein concentration from the 25 individuals with healthy periodontium who were used as negative controls in the test. Five wells of 20 $\mu\text{g}/\text{mL}$ rPrtC were used as the positive control.

Statistical analysis The clinical and laboratory data were presented as mean \pm SD. Student's *t*-test and ANOVA were used to determine the significance of the differences between the sub-groups. $P < 0.05$ was considered statistically significant.

Results

PCR detection of *P. gingivalis* in subgingival plaque samples The 25 individuals with healthy periodontium were all negative for *P. gingivalis*. In the 196 subgingival samples from CP patients, 95.9% (188/196) and 91.8% (180/196) of the samples were positive for *P. gingivalis* *16SrDNA* and *prtC*, respectively. None of the samples were positive for *prtC* and negative for *16SrDNA* (Figure 1).

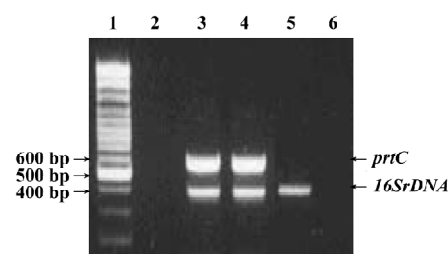


Figure 1. Target amplification fragments of *P. gingivalis* *16SrDNA* and *prtC* genes in subgingival plaque samples. Lane 1, 100 bp DNA marker; lane 2, blank control; lane 3, positive control of *P. gingivalis* strain ATCC 33277; lane 4, a subgingival plaque sample with the 2 different target fragments amplified from the *16SrDNA* and *prtC* genes, respectively; lane 5, a subgingival plaque sample with the 1 target fragment alone that was amplified from the *16SrDNA* gene; lane 6, a subgingival plaque sample with negative PCR results for both the *16SrDNA* and *prtC* genes.

Nucleotide sequence analysis The target fragments with the expected size amplified from genomic DNA of *P. gingivalis* strain ATCC 33277 are shown in Figure 2. Compared with the published sequences (GenBank Accession No. AB006973),

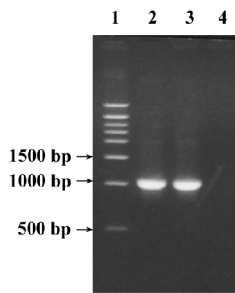


Figure 2. Target *prtC* gene fragments amplified from genomic DNA of *P. gingivalis* strains. Lane 1, 500 bp DNA marker; lanes 2 and 3, the target fragments amplified from genome DNA of *P. gingivalis* strains ATCC 33277 and 47A-1, respectively; lane 4, blank control.

the homology of the nucleotide and putative amino acid sequences of the amplification fragment were 98.46% and 99.07%, respectively.

Expression of target fusion protein IPTG 0.5 mmol/L was able to efficiently induce rPrtC expression (Figure 3). Purified rPrtC showed 1 single band on 10% SDS-PAGE. rPrtC was mainly presented in ultrasonic precipitation and the output was approximately 50% of the total bacterial proteins.

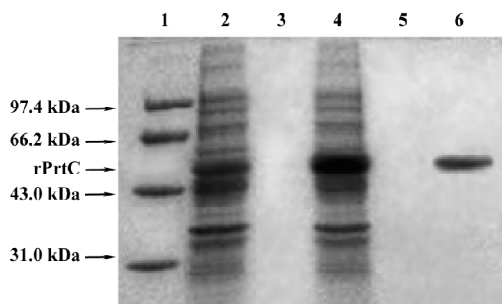


Figure 3. rPrtC expression and purity of rPrtC extract. Lane 1, protein molecular marker (Shanghai Shisheng); lane 2, non-induced; lanes 3 and 5, blank; lane 4, induced with 0.5 mmol/L IPTG; lane 6, purified rPrtC by Ni-NTA affinity chromatography.

Antigenicity and immunoreactivity of rPrtC The rabbit anti-rPrtC antisera was found positive for *P. gingivalis* ATCC 33277 by slide agglutination reaction; the A_{490} value of the supernatants was 0.89 ± 0.10 as determined by ELISA. The slide agglutination reactions with the other 5 species were all negative; the A_{490} value was $0.01 \sim 0.03 \pm 0.02 \sim 0.03$, which indicated that the antisera were specific. Furthermore, Western blotting indicated that rPrtC could combine with the rabbit anti-rPrtC serum and rabbit antiserum against the whole cell of *P. gingivalis*, and only one distinct band was shown

(Figure 4). It demonstrated that rPrtC could induce rabbit to produce specific antiserum, showing good antigenicity of the recombinant protein. The fact rPrtC could combine with these antiserum also implied its immunoreactivity.

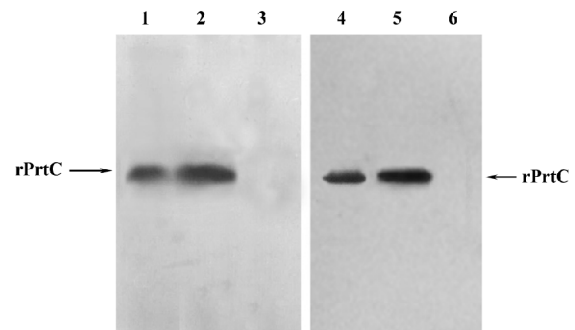


Figure 4. Western blotting detecting rPrtC with rabbit anti-rPrtC or anti-*P. gingivalis* antiserum. Lanes 1 and 2, loading volumes of 20 and 30 μ L of purified rPrtC fragments reacted with rabbit anti-rPrtC serum, respectively; lanes 3 and 6, blank controls; lanes 4 and 5, loading volumes of 20 and 30 μ L of purified rPrtC fragments reacted with rabbit antiserum against the whole cell of *P. gingivalis*, respectively.

Effects of rPrtC on the secretion of inflammatory cytokines in ECV304 cells After the ECV304 cells were treated with 1 μ g/mL rPrtC for 24 h or with 5 and 10 μ g/mL rPrtC for 12 h, the secretion of IL-1 α , IL-8 and TNF- α in the supernatants increased significantly ($P > 0.05$), among which the IL-1 α level peaked at 24 h and the IL-8 and TNF- α levels increased gradually with time. Slightly higher levels of IL-1 α and TNF- α were shown in the ECV304 cells stimulated with 5 or 10 μ g/mL rPrtC than with 1 μ g/mL rPrtC for the same time intervals (Figure 5).

PrtC level in subgingival plaque samples before and after treatment The mean \pm SD at A_{490} of the negative sulcular samples was 0.06 ± 0.06 , and the positive reference value was 0.24. According to this, 90.8% (178/196) of the subgingival plaque samples were positive for PrtC with an A_{490} value ranging from 0.26 to 1.23. All these 178 PrtC positive results were from *prtC* gene positive samples. Only 2 samples were PrtC negative, but were *prtC* gene positive. The correlation between the levels of *P. gingivalis* PrtC in subgingival plaques and clinical parameters are shown in Table 1. The A_{490} value in the BOP-positive sites was significantly higher than that in BOP-negative sites ($P = 0.029$). The enhanced level of PrtC was found in the ≥ 5 mm AL sites than that in the ≤ 2 mm AL sites ($P = 0.016$), but with no significant difference between the ≥ 5 and $>2 - <5$ mm AL sites or between the ≤ 2

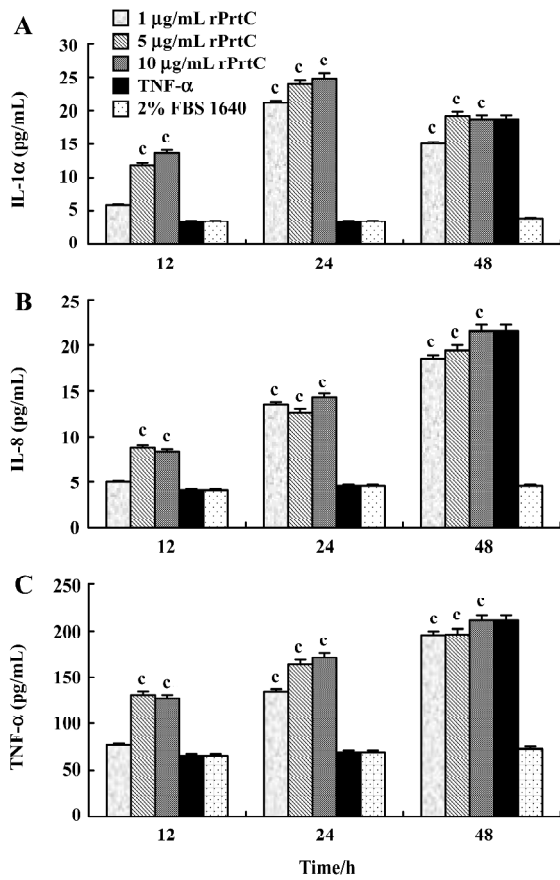


Figure 5. Secretion of inflammatory cytokines of human umbilical vein endothelial cell ECV304 induced by rPrtC. Cells were treated with 1, 5, or 10 μg/mL rPrtC for 0, 12, 24 or 48 h. The concentrations of (A) IL-1α, (B) IL-8 and (C) TNF-α in supernatants increased significantly after incubation with rPrtC. Mean±SD. *n*=3. ^c*P*<0.01 vs negative controls.

and >2–<5 mm AL sites (*P*=0.114, 0.362). A higher *A*₄₉₀ value was detected in the >6 mm PD pockets than in >4–≤6 mm or in the 3–4 mm sites (*P*=0.039, 0.023). No statistical difference could be distinguished between the >4–≤6 mm and the 3–4 mm pockets (*P*=0.506).

Overall, the periodontal conditions in the periodontitis patients were improved significantly 4 months after the initial periodontal treatment (Table 2). Only 56 of the 157 sites were still positive for PrtC (Table 1). The *A*₄₉₀ value decreased after treatment, with 66.8% (131/196) of the samples positive for PrtC in a range of 0.25–0.88. Although the PrtC level in the BOP-positive sites was still higher than that in the BOP-negative sites after treatment (*P*=0.004), no significant difference was found between the *A*₄₉₀ values before and after treatment (*P*=0.261, 0.286). Higher values were detected in the ≥5 mm AL sites than those in ≤2 mm AL sites (*P*=0.011),

Table 1. Correlation between level of *P. gingivalis* PrtC in subgingival plaques samples and clinical parameters before and after initial periodontal treatment (*A*₄₉₀). Mean±SD. ^b*P*<0.05 or ^c*P*<0.01 vs values from sites with BOP negative, AL≤2 mm, PD≤6 mm. ^e*P*<0.05 vs values before treatment.

Groups	<i>A</i> ₄₉₀ values for PrtC before (cases)	<i>A</i> ₄₉₀ values for PrtC after (cases)
BOP		
Positive	0.47±0.18 (157) ^b	0.44±0.18 (56) ^c
Negative	0.40±0.17 (39)	0.37±0.14 (140)
AL		
≤2 mm	0.42±0.17 (52)	0.36±0.14 (75) ^c
>2 mm–<5 mm	0.45±0.18 (64)	0.39±0.16 (66) ^c
≥5 mm	0.50±0.19 (77) ^b	0.43±0.17 (55) ^{ce}
PD		
3–4 mm	0.42±0.15 (49)	0.35±0.14 (71) ^c
>4 mm–≤6 mm	0.44±0.17 (73)	0.37±0.15 (69) ^c
>6 mm	0.50±0.21 (74) ^b	0.46±0.17 (56) ^c
Controls		
Positive	1.14±0.26 (5)	–
Negative	0.06±0.06 (25)	–

BOP: bleeding on probing, AL: attachment loss, PD: probing depth.

Table 2. Changes in clinical parameters in CP patients before and after treatment. Mean±SD. ^b*P*<0.05 vs values before treatment.

Variables	Before	After
Age (years)	45.0±8.2	45.0±8.2
Gender (male/female)	24/25	24/25
mean BOP (%)	57.51±10.50	29.79±4.43 ^b
mean PD (mm)	3.86±0.54	2.81±0.46 ^b
mean AL (mm)	4.13±0.31	3.19±0.25 ^b

BOP: bleeding on probing, AL: attachment loss, PD: probing depth.

but with no statistical difference between the ≥5 and the >2–<5 mm AL sites or between the ≤2 and the >2–<5 mm AL sites (*P*=0.186, 0.237). A remarkably enhanced level of PrtC was observed in the >6 mm PD pockets than that in the >4–≤6 mm or in the 3–4 mm pockets (*P*=0.002, 0.000), but the *A*₄₉₀ values between the >4–≤6 mm and the 3–4 mm PD pockets were almost similar (*P*=0.416). After treatment, the PrtC level in the different AL groups or the ≤6 mm PD groups decreased markedly compared with that before treatment (*P*=0.010–0.047). Although the *A*₄₉₀ values also dropped slightly compared with that before treatment in the sites with >6 mm PD, no statistical difference could be found (*P*=0.246).

Discussion

In the present study, a prokaryotic expression system was constructed to obtain plentiful purified recombinant PrtC, which would be helpful in investigating the potential role of *P gingivalis* collagenase in periodontal destruction, because it was difficult to extract a great deal of purified collagenase directly from *P gingivalis*. A nucleotide sequence analysis showed 98.46% and 99.07% homology of the nucleotide and putative amino acid sequences of our cloned *prtC* gene compared with what was registered in GenBank, indicating high fidelity of the method. A low dosage of 0.5 mmol/L IPTG could efficiently induce rPrtC expression with a 50% output of the bacterial total proteins, suggesting the efficiency of the constructed prokaryotic expression system. The fact that rPrtC could be recognized by the antibody against the whole cell of *P gingivalis* and that it was able to induce rabbit antisera to produce the specific antibody, demonstrated good immunoreactivity and antigenicity of the recombinant protein. A high frequency of infection of *P gingivalis* in the subgingival plaque sample in CP patients (95.9%) was revealed by PCR. In the 196 samples, 188 and 180 were positive for *P gingivalis* 16SrDNA and *prtC*, respectively. The data implied that the *prtC* gene might be present in most *P gingivalis* strains (180/188), which was consistent with previous reports^[25,26]. Furthermore, compared with the PCR detection of the *prtC* gene, our established ELISA was highly sensitive. Among the 180 samples positive for the *prtC* gene, 178 were found to be PrtC positive by ELISA. This indicated that the *prtC* gene was frequently expressed in this species.

This is the first study to demonstrate that rPrtC is able to directly promote the production of IL-1 α , IL-8 and TNF- α in endothelial cells. Initial studies indicated that the *prtC* gene product could cleave type I collagen, suggesting that this enzyme might play a role in connective tissue destruction in periodontitis^[4,11]. More recent findings however questioned the role of the *prtC* gene product in the collagenolytic activity of *P gingivalis*^[27,28]. Some researchers have found that proteinase from some bacteria could have direct inflammation-causing ability on host cells besides its specific enzyme activity^[13,29]. However, studies of the influence of the *prtC* gene product of *P gingivalis* on host cells are still lacking. On the other hand, neutrophil-endothelial cell interactions are the prerequisite for the transendothelial migration of leukocytes, activation of T cells, and then establishment of local inflammation^[29], so endothelial cells play a critical role in the development of inflammation. Moreover, endothelial cells may be important target cells for periodontal pathogens, including *P gingivalis*^[17]. In this study, we investigated the

effect of recombinant PrtC on the secretion of cytokines in ECV304 endothelial cells. After co-incubation with 1 μ g/mL rPrtC for 24 h and with 5 or 10 μ g/mL rPrtC for 12 h, the levels of IL-1 α , IL-8, and TNF- α increased significantly ($P < 0.05$). IL-1 α and TNF- α exert multiple effects on inflammatory cells, including the synthesis of other cytokines and the modulation of intracellular adhesion molecule-1 upregulation, a critical step in the development of neutrophil-endothelial cell interactions^[13,19,29]. IL-8 is key mediator of neutrophil migration to sites of inflammation^[13,19]. IL-8 is produced in response to cytokines such as TNF- α , IL-1, and LPS^[13,19]. So the ability of PrtC to induce the expression of these cytokines in endothelial cells implies an important role of PrtC in the inflammation of periodontal tissue. Much attention has been given to proteinase produced by host cells. MMP, enzymes secreted by endothelial cells, macrophages, and fibroblasts, are known to be involved in tissue remodeling, organogenesis, angiogenesis, wound repair, and inflammatory cellular infiltration by degrading extracellular matrix^[16,30]. At the inflammatory site, inflammatory cells are mobilized and MMP are produced as pro-enzymes and activated by various stimulatory factors^[30]. Recent studies demonstrated that MMP expression was upregulated by pro-inflammatory mediators including IL-1 and TNF- α ^[16]. Taken together, these findings suggest that although the *prtC* gene product may not be a true bacterial collagenase, it could have a strong effect on inducing host cells to secrete inflammatory cytokines and further enhance the collagenase activity of host cells such as MMP, which lead to the destruction of periodontal connective tissue.

Our data further demonstrated that the PrtC level in clinical samples was correlated with BOP, AL, and PD before and after treatment (Table 1). ELISA, with the self-prepared rabbit anti-rPrtC serum, was established to measure the PrtC level in subgingival samples to understand the association between PrtC and periodontal inflammation or tissue destruction. The PrtC level was higher in the BOP-positive sites or in serious periodontal destruction sites (≥ 5 mm AL) than in the BOP-negative or in the ≤ 2 mm AL sites ($P < 0.05$). The A_{490} value of PrtC was higher in the deep pockets (> 6 mm PD) than that in shallow or moderate pockets ($P < 0.05$). This indicates that the PrtC level is related to periodontal inflammation and tissue destruction. A previous study in domestic cats showed that serum antibody response to the recombinant PrtC of a feline strain of *P gingivalis* was associated with the severity of periodontal disease^[12]. The present *in vivo* study provided strong evidence that PrtC correlated with periodontal tissue destruction and inflammation. PrtC might play a role in the initiation and development of peri-

odontitis by working in combination with other factors produced by *P gingivalis* or host cells, including *P gingivalis* LPS or MMP^[31,32], since the relationships between PrtC levels and these clinical parameters seem to be rather complicated. It was notable that the PrtC-positive subgingival samples were reduced from 90.8% to 66.8%, and the PrtC level decreased remarkably in different AL sites or in the ≤6 mm PD pockets ($P < 0.05$) after treatment, which might be explained by the fact that mechanical debridement will significantly decrease the number of *P gingivalis* and other putative periodontal pathogens^[32,33]. However, the A_{490} value of PrtC in the BOP-positive samples and in the sites with >6 mm PD changed insignificantly ($P > 0.05$). Previous studies have indicated that deep pockets and furcations are most likely inadequately instrumented^[34] and microorganisms, including *P gingivalis* residing in biofilms left in such locations, correlated with BOP and greater pocket depths^[35], which may require surgical intervention^[32]. Our results also contributed to the above viewpoints.

In conclusion, periodontal diseases are multifactorial infections elicited by a complex of bacterial species that interact with host tissues and cells, which lead to secretion of various cytokines, including IL-1, IL-6, IL-8, and TNF- α , as well as prostaglandin E₂ and MMP^[2,3,8]. These cytokines and enzymes play critical roles in periodontal tissue destruction. The results obtained in this study demonstrated that the *prtC* gene product could induce host cells to synthesize and secrete inflammatory cytokines to exert a destructive effect on periodontal tissue. Further investigation is needed to reach a conclusive result.

Acknowledgement

We would like to thank Dr Wei-lian SUN for assistance in the recruitment of patients and the collection of clinical samples.

References

- 1 Kumagai Y, Yagishita H, Yajima A, Okamoto T, Konishi K. Molecular mechanism for connective tissue destruction by dipeptidyl aminopeptidase IV produced by the periodontal pathogen *Porphyromonas gingivalis*. *Infect Immun* 2005; 73: 2655–64.
- 2 Socransky SS, Haffajee AD. Dental biofilms: difficult therapeutic targets. *Periodontol 2000* 2002; 28: 12–55.
- 3 Bodet C, Chandad F, Grenier D. Inflammatory responses of a macrophage/epithelial cell co-culture model to mono and mixed infections with *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*. *Micro Infect* 2006; 8: 27–35.
- 4 Beikler T, Ehmke B, Wittstock M, Schmidt H, Karch H, Flemmig TF. Serum antibody reactivity against recombinant PrtC of *Porphyromonas gingivalis* following periodontal therapy. *J Periodont Res* 2003; 38: 276–81.
- 5 Chen LL, Wu YM, Yan J, Sun WL, Sun YZ, Ojcius D. Association between coinfection of *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Treponema denticola* and periodontal tissue destruction in chronic periodontitis. *Chin Med J (Engl)* 2005; 118: 915–21.
- 6 Cortelli JR, Cortelli SC, Jordan S, Haraszthy VI, Zambon JJ. Prevalence of periodontal pathogens in Brazilians with aggressive or chronic periodontitis. *J Clin Periodontol* 2005; 32: 860–6.
- 7 Haffajee AD, Socransky SS, Smith C, Dibart S. Relation of baseline microbial parameters to future periodontal attachment loss. *J Clin Periodontol* 1991; 18: 744–50.
- 8 Holt SC, Kesavalu L, Walker S, Genco CA. Virulence factors of *Porphyromonas gingivalis*. *Periodontol 2000* 1999; 20: 168–238.
- 9 Bedi GS, Williams T. Purification and characterization of a collagen-degrading protease from *Porphyromonas gingivalis*. *J Biol Chem* 1994; 269: 599–606.
- 10 Takahashi N, Kato T, Kuramitsu HK. Isolation and preliminary characterization of the *Porphyromonas gingivalis prtC* gene expressing collagenase activity. *FEMS Microbiol Lett* 1991; 84: 135–8.
- 11 Kato S, Takahashi N, Kuramitsu HK. Sequence analysis and characterization of the *Porphyromonas gingivalis prtC* gene, which expresses a novel collagenase activity. *J Bacteriol* 1992; 174: 3889–95.
- 12 Norris JM, Love DN. The association of two recombinant proteinases of a feline strain of *Porphyromonas gingivalis* with periodontal disease in cats. *Vet Microbiol* 2000; 71: 69–80.
- 13 Tanahashi T, Kita M, Kodama T, Yamaoka Y, Sawai N, Ohno T, *et al*. Cytokine expression and production by purified *Helicobacter pylori* urease in human gastric epithelial cells. *Infect Immun* 2000; 68: 664–71.
- 14 Papapanou PN, Sandros J, Lindberg K, Duncan MJ, Niederman R, Nannmark U. *Porphyromonas gingivalis* may multiply and advance within stratified human junctional epithelium *in vitro*. *J Periodont Res* 1994; 29: 374–5.
- 15 Lamont RJ, Chan A, Belton CM, Izutsu KT, Vasel D, Weinberg A. *Porphyromonas gingivalis* invasion of gingival epithelial cells. *Infect Immun* 1995; 63: 3878–85.
- 16 Zhou J, Windsor L. *Porphyromonas gingivalis* affects host collagen degradation by affecting expression, activation, and inhibition of matrix metalloproteinases. *J Periodont Res* 2006; 41: 47–54.
- 17 Walter C, Zahlten J, Schmeck B, Schaudinn C, Hippenstiel S, Frisch E, *et al*. *Porphyromonas gingivalis* strain-dependent activation of human endothelial cells. *Infect Immun* 2004; 72: 5910–8.
- 18 Hack CE, Zeerleder S. The endothelium in sepsis: source of and a target for inflammation. *Crit Care Med* 2001; 29: S21–7.
- 19 Zhou Q, Desta T, Fenton M, Graves DT, Amar S. Cytokine profiling of macrophages exposed to *Porphyromonas gingivalis*, its lipopolysaccharide, or its FimA protein. *Infect Immun* 2005; 73: 935–43.
- 20 Yang QW, Zhu PF, Wang ZG, Jiang JX. Lipopolysaccharide upregulates the expression of Toll-like receptor 4 in human vas-

- cular endothelial cells. *Chin Med J (Engl)* 2002; 115: 286–9.
- 21 Dong YG, Chen DD, He JG, Guan YY. Effects of 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ on cell proliferation and apoptosis in ECV304 endothelial cells. *Acta Pharmacol Sin* 2004; 25: 47–53.
 - 22 Jiang JL, Jiang DJ, Tang YH, Li NS, Deng HW, Li YJ. Effect of simvastatin on endothelium-dependent vaso-relaxation and endogenous nitric oxide synthase inhibitor. *Acta Pharmacol Sin* 2004; 25: 893–901.
 - 23 Armitage GC. Development of a classification system for periodontal disease and conditions. *Ann Periodontol* 1999; 4: 1–6.
 - 24 Slots J, Ashimoto A, Flynn MJ, Li G, Chen C. Detection of putative pathogens in subgingival specimens by 16S ribosomal DNA amplification with the polymerase chain reaction. *Clin Infect Dis* 1995; 20 (Suppl 2): 304–7.
 - 25 Bodinka AH, Schmidt B, Henkel TF, Flemmig TF, Klaiber B, Karch H. Polymerase chain reaction for the identification of *Porphyromonas gingivalis* collagenase genes. *Oral Microbiol Immunol* 1994; 9: 161–5.
 - 26 Slots J, Flynn MJ, Li G. Polymerase chain reaction analysis of the *Porphyromonas gingivalis* collagenase gene. *Clin Infect Dis* 1995; 20 (Suppl 2): S167–8.
 - 27 Kuramitsu HK, Yoneda M, Madden T. Proteases and collagenases of *Porphyromonas gingivalis*. *Adv Dent Res* 1995; 9: 37–40.
 - 28 Lamont RL, Jenkinson HF. Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. *Microbiol Molec Biol Rev* 1998; 62: 1244–63.
 - 29 Innocenti M, Thoreson AC, Ferrero RL, Stromberg E, Bolin I, Eriksson L, *et al*. *Helicobacter pylori*-induced activation of human endothelial cells. *Infect Immun* 2002; 70: 4581–90.
 - 30 Lee SW, Song KE, Shin DS, Ahn SM, Ha ES, Kim DJ, *et al*. Alterations in peripheral blood levels of TIMP-1, MMP-2, and MMP-9 in patients with type-2 diabetes. *Diabetes Res Clin Pract* 2005; 69: 175–9.
 - 31 Chen LL, Yan J. *Porphyromonas gingivalis* lipopolysaccharide activated bone resorption of osteoclasts by inducing IL-1, TNF, and PGE. *Acta Pharmacol Sin* 2001; 22: 614–8.
 - 32 Sigusch BW, Guntsch A, Pfitzner A, Glockmann E. Enhanced root planning and systemic metronidazole administration improve clinical and microbiological outcomes in a two-step treatment procedure. *J Periodontol* 2005; 76: 991–7.
 - 33 Loesche WJ, Grossman NS. Periodontal disease as a specific, albeit chronic, infection: diagnosis and treatment. *Clin Microbiol Rev* 2001; 14: 727–52.
 - 34 Serino G, Rosling B, Ramberg P, Hellstrom MK, Socransky SS, Lindhe J. The effect of systemic antibiotics in the treatment of patients with recurrent periodontitis. *J Clin Periodontol* 2001; 28: 411–8.
 - 35 Kasuga Y, Ishihara K, Okuda K. Significance of detection of *Porphyromonas gingivalis*, *Bacteroides forsythus* and *Treponema denticola* in periodontal pockets. *Bull Tokyo Dent Coll* 2000; 41: 109–17.