Construction and characterization of a *Porphyromonas gingivalis* htpG disruption mutant

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Abstract

Our previous reports implicated the Hsp90 homologue (HtpG) of *Porphyromonas gingivalis* (Pg) in its virulence in periodontal disease. We investigated the role of the HtpG stress protein in the virulence of *Pg*. This report describes the (i) expression of a recombinant *Pg* HtpG (rHtpG), (ii) generation and characterization of a polyclonal rabbit anti-*Pg* rHtpG antiserum, and (iii) construction of a *Pg* htpG isogenic mutant and evaluation of the growth, adherence and invasion properties compared to the wild-type parental strain. The disruption of the *htpG* gene did not significantly affect growth, and had no effect on *Pg* adherence to and invasion of cultured human cells.

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1. Introduction

In an earlier epidemiological study of periodontal disease, we reported that serum antibodies reactive with human Hsp90 stress protein were correlated with periodontal health when *Porphyromonas gingivalis* (*Pg*) was present in the sub-gingival plaque [1]. In previous reports, we described the presence of a 68-kDa protein (HtpG) in *Pg*, the Hsp90 homologue, the cloning of the gene, and demonstrated its surface localization and cross-reactivity with human Hsp90 [2], [3]. We hypothesized that the HtpG protein of *Pg* contributed to the virulence of the microorganism.

Associations between microbial virulence and stress protein expression have been identified and attributed to molecular mimicry of the prokaryotic Hsps [4], recognition of Hsps by pathogen-associated molecular pattern receptors [5], [6], and adherence and invasion mechanisms [7]. However, very little is known about the role of the prokaryotic HtpG (Hsp90 family) in microbial virulence.

This report introduces our investigation into the role of the HtpG protein in the virulence of *Pg* through the construction of an *htpG* isogenic mutant and the assessment of the effects the mutation has on adherence to and invasion of cultured human cells.

2. Materials and methods

2.1. Bacterial strains and cultivation

*Pg* (ATCC 33277, SUNYaB A7A1-28, ATCC 53978 (W50), W83 and 381) and *Actinobacillus actinomycetemcomitans* (Aa, ATCC 43718) were grown under anaerobic conditions at 37°C. Cultures were maintained on blood agar plates (BAPs, enriched trypticase soy broth +5% defibrinated sheep blood) or anaerobic Medium 1 plates (Remel). Broth cultures were grown in BH broth (BBL) or mycoplasma broth base (BBL) supplemented with 5 μg mL⁻¹ menadione and 5 μg mL⁻¹ hemin. The *Pg* htpG disruption mutant, *Pg* W83 ΔhtpG, was maintained on BAPs with 10 μg mL⁻¹ erythromycin (Sigma). *Pg* cultures designated as ‘heat stressed’ were incubated at 45°C under anaerobic conditions.
aerobic conditions for 1 h, unless otherwise indicated. Matched control samples were maintained at 37°C.

*Escherichia coli* DH5α was grown aerobically in Luria–Bertani (LB) medium at 37°C. *E. coli* JB45, an *htpG* deletion mutant [8], was grown in media supplemented with 100 μg ml⁻¹ kanamycin (Sigma). *E. coli* JM109, used as a host strain in mutant construction [9], was grown in LB broth or agar medium supplemented with ampicillin (50 μg ml⁻¹), kanamycin (50 μg ml⁻¹) or Erythromycin (200 μg ml⁻¹) as appropriate. *E. coli* M15 [pREP4] (Qiagen) was grown in media supplemented with kanamycin (25 μg ml⁻¹) to maintain the control plasmid pREP4. *E. coli* M15 [pREP4] transforms containing the pDGS03 (pQE30::*Pg htpG*) plasmid (see below) were grown in media containing both 25 μg ml⁻¹ kanamycin and 100 μg ml⁻¹ of ampicillin (Sigma).

### 2.2. DNA isolation

Genomic DNA was isolated from bacteria using the Wizard® Genomic DNA Isolation Kit (Promega) per manufacturer's instruction and stored at 4°C until used.

### 2.3. Expression of recombinant *Pg* HtpG

*Pg* htpG was used in the construction of the expression plasmid pQE30::*Pg htpG*, or pDGS03 [3]. *Pg* htpG was cloned into the commercial expression vector, pQE30, using *HindIII* and *KpnI* restriction sites to facilitate directional cloning with a modified start codon to facilitate incorporation of a poly-His N-terminal tag (Qiagen). Briefly, an amplicon was generated by polymerase chain reaction (PCR) using *Tag* DNA polymerase (PCR SuperMix; Invitrogen) with *Pg* ATCC 33277 DNA as a template and the oligonucleotide primers: 77521 (5'GGG TAC CAT AAG TAA GAA GCT TGC AAT CGG GGT AAC G-3') and EO4 (5'GGC CAA GCT TGC AAT CGG GGT AAC G-3'). The insert placement, reading frame, and sequence were confirmed by double-strand sequencing at the University of Michigan Biomedical Research Core Facilities using the pQE30 primers supplied by the manufacturer (Qiagen). Recombinant *Pg* HtpG (Pg rHtpG) was expressed and purified using the QIAexpress Kit Type IV (Qiagen) per manufacturer’s instructions and frozen at −20°C.

### 2.4. Antibody production/characterization

Polyclonal rabbit antibodies to the *Pg* rHtpG protein were prepared commercially (Lampire Biological Laboratories). Complete Freund’s adjuvant was used during primary immunization; subsequent booster immunization employed incomplete Freund’s adjuvant. The pre-immune serum and subsequent test bleeds were assessed by enzyme-linked immunosorbent assay (ELISA) and Western immunoblotting to determine specificity and titer of the antibody. Cross-reactive antibodies were removed by absorption on a column of whole formalin-fixed *E. coli* cells.

### 2.5. Western immunoblotting

Western immunoblotting was performed as previously described [3]. Briefly, bacterial extracts were prepared by 10% trichloroacetic acid precipitation. After determining the protein concentration (BioRad Protein Assay Reagent, Bio-Rad Laboratories) the extracts were boiled in NuPage LDS Sample Buffer (Novex), resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electrophotically transferred to nitrocellulose membranes. The blots were probed with rabbit anti-human Hsp90 or anti-*Pg* rHtpG antibodies and detected by chemiluminescence (ECL, Amersham) using horseradish peroxidase-conjugated goat anti-rabbit IgG antibody.

### 2.6. ELISA

*Pg* rHtpG (5 μg ml⁻¹) in carbonate coating buffer, 100 μl per well, was added to each well of a clear 96-well flat-bottom microELISA plates (Immunlon 1, Dynatech Laboratories, Inc.) and incubated for 30 min at room temperature. After washing the wells with phosphate-buffered saline-Tween (PBS-T), 100 μl per well of serially diluted (1:10 to 1:1 000 000 in PBS-T) anti-*Pg* rHtpG antisera was added and incubated for 1 h at RT. The plates were washed with PBS-T and 100 μl per well alkaline phosphatase-conjugated goat anti-rabbit IgG (1:1000 in PBS-T) was added and incubated for 1 h at RT. After washing with PBS-T, Sigma 104 phosphatase substrate (Sigma) was added to each well, and color intensity measured at 405 nm after development.

### 2.7. Mutagenesis of *Pg* htpG

An internal fragment of *Pg htpG* was PCR-amplified from *Pg* genomic DNA using oligonucleotide primers 74501 (5'GGG TAC CAT AAG TAA GAA GCT TGC AAT CGG GGT AAC G-3') and 74511 (5'GGC CAA GCT TGC AAT CGG GGT AAC G-3'). The amplicon was digested in EcoRI yielding a 1.5-kb *htpG* fragment that was gel purified and cloned in pBluescript KSII+, yielding pBHT1. pBHT1 was linearized with *BglII* (nucleotide position 1019 of the *htpG* coding sequence [3],) blunted with Klenow fragment DNA polymerase I and ligated to blunt-ended *ermFlAM* gene cassette [10], yielding pBHT4 (Fig. 1A). The *ermFlAM* gene cassette and the *htpG* fragments of pBHT4 were determined to be in the same transcriptional orientation. For mutagenesis, pBHT4 was digested to completion in EcoRI and introduced into *Pg* W83 by electroporation [10]. Following overnight recovery in antibiotic-free medium, erythromycin-resistant colonies were recovered on BAPs containing 0.5 μg ml⁻¹ erythromycin, and then grown in broth containing 5 μg ml⁻¹ erythromycin.
The construction of the allelic replacement mutant was confirmed by PCR analysis of genomic DNA (data not shown), and the loss of expression of HtpG protein was confirmed by Western immunoblotting using rabbit anti-

2.8. Cell culture

All human cell cultures were maintained at 37°C, 5% CO2, and 80–90% humidity. KB cells (ATCC CCL-17) were grown in DMEM (Invitrogen) with 10% horse serum and 100 μg ml−1 gentamycin (Sigma). HGF cells (human gingival fibroblasts, a generous gift from Dr. Paul Krebsbach) were grown in α-MEM (Invitrogen) with 10% fetal bovine serum (FBS) and 100 μg ml−1 penicillin-streptomycin (Sigma). THP-1 cells (ATCC TIB-202) were grown in RPMI (Invitrogen) with 10% FBS and 100 μg ml−1 penicillin-streptomycin.

2.9. Measurement of adherence and invasion of Pg into human cells

*Pg* W83 and *Pg* W83 ΔhtpG were grown to mid-exponential phase (OD600 = 0.4–0.6), pelleted for 10 min at 1000×g, and resuspended in antibiotic-free media containing 1% serum. The mammalian cells described above in Section 2.8, 50–80% confluent, were washed with antibiotic-free media containing 1% serum. The type of serum used varied as appropriate for the cell line as described above. Bacteria were added to the washed cells (100:1, bacteria: cells, unless otherwise stated) and incubated at 37°C for 60 min. Determination of adherence and invasion was performed as described elsewhere [11]. Briefly, following the 60 min incubation, the cells were washed to remove non-adherent bacteria and then either fresh media (total bacteria adhering plus invaded) or fresh media containing 300 μg ml−1 gentamycin (bacteria invaded only) was added and incubated for 60 min. The cell cultures were then lysed with distilled water and plated on BAPs, or the RNA was harvested with Trizol (Invitrogen) for real-time quantitative PCR analysis using *Pg* specific 16S RNA primers [12].

2.10. Quantization of bacteria by fluorescence

A method that quantifies total nucleic acid in a sample using a DNA binding dye (SYBR Green I, Molecular Probes) was adapted to measure the growth of bacteria [13]. Three milliliters of 10% (v/v) formaldehyde in 10 mM PO4 buffer (pH 7.5) was added to 30-μl aliquots of bacteria in a black, flat-bottomed 96-well plate (Costar). SYBR Green I (175 μl of a 1:10000 dilution of the stock) was then added to the bacterial suspensions. After incubation at room temperature for 45 min the fluorescence was measured (excitation 485 nm, emission 535 nm). Preliminary experiments demonstrated that the method correlated with turbidimetric (OD600) estimates with an r value of 0.978. All subsequent measurements were expressed either as the percent of the untreated control cultures or as the actual concentration by the use of standards calibrated by turbidimetric measurements.

2.11. Statistical and in silico analyses

Statistical analyses were performed with the Statistica software package, v6.0.

3. Results

3.1. *Pg* rHtpG expression and reactivity of polyclonal anti-*Pg* rHtpG

Due to the significant protease activity of wild-type *Pg* and the labile nature of the native protein, rHtpG was expressed in *E. coli*. A poly-His N-terminal tag facilitated purification of the fusion protein. As demonstrated by SDS–PAGE (Fig. 2A) induction of expression of rHtpG with isopropyl-beta-D-thiogalactopyranoside (IPTG) resulted in significant levels of protein at 78 kDa. This protein was used to immunize rabbits to prepare polyclonal anti-*Pg* rHtpG.

Western immunoblot analysis was used to demonstrate reactivity of the polyclonal rabbit anti-*Pg* rHtpG (Fig. 1B).
2B.C). In preliminary assessment of the antibody, it was determined that the antibody had an effective titer of at least 1:1,000,000 against Pg rHtpG in Western immunoblots, however the antibodies were used routinely at a dilution of 1:100,000. Pre-immune serum had no reactivity against this protein (data not shown). As shown, there was no detectable reactivity against either E. coli or A. actinomycetemcomitans HtpG (Fig. 2B, lanes 1, 2, 4). Heat stress induction could be shown in wild-type Pg (Fig. 2B, lanes 5 vs. 6). However, a minor band at approximately 60 kDa could be visualized and corresponded to E. coli GroEL, which probably bound to Pg rHtpG during expression or extraction (data not shown). This reactivity was easily absorbed from the antibody using whole formalin-fixed E. coli cells. E. coli JB45 is an htpG deletion mutant [8] included as a negative control for reactivity to the anti-Pg rHtpG antiserum, and E. coli DH5α is the wild-type strain.

Immunoreactivity of whole cell extracts from the five isolates of Pg (ATCC 33277, SUNYaB A7A1-28, ATCC 53978 (W50), W83 and 381) with the anti-Pg rHtpG antiserum was assessed (Fig. 2C). The recombinant protein was included as a positive control. In all cases, we were able to demonstrate basal expression of native HtpG, which was upregulated following heat stress at 45°C. All strains reacted with a single band at a molecular size of 78 kDa.

3.2. Growth characteristics of the Pg W83 ΔhtpG

In construction of the Pg W83 ΔhtpG, there were concerns that there might be growth effects that might affect future animal model studies. As shown in Fig. 3, while the disruption of the htpG had minimal early effects on growth, by 60 h, the mutant and parental strains were indistinguishable.

3.3. Loss of HtpG does not compromise adherence or invasion of cultured cells

As a potential virulence determinant, we wanted to evaluate the effect of deleting the production of HtpG on the ability of Pg to adhere and invade epithelial cells. Therefore, we compared the abilities of Pg W83 and Pg W83 ΔhtpG to adhere and invade a cultured human cell line, using an established invasion protocol for Pg [14]. There was no statistically significant difference in the ability of Pg W83 ΔhtpG to adhere or invade the cultured human cell line when compared to the Pg W83 parent strain. In a typical experiment, 0.05 ± 0.01 Pg W83 parent strain cells adhered per KB cell, while 0.06 ± 0.05 Pg W83 ΔhtpG cells adhered per KB cell. Likewise, 0.02 ± 0.003 Pg W83 parent strain cells invaded per KB cell and 0.03 ± 0.001 Pg W83 ΔhtpG cells invaded per KB cell. These data are consistent with results of other adherence and invasion experiments with Pg [14].

4. Discussion

It was not practical to directly isolate HtpG from Pg due to the amount and extent of protease activity associated with this microorganism [3]. The htpG gene was cloned into an expression system employing a 6×-His tag which could be used to purify the fusion protein. The expression system did not incorporate an endokinase cleavage site to facilitate removal of the poly-His tags. Thus, the poly-His tag remained on the N-terminus of the purified Pg rHtpG protein. The poly-His moiety is known to be of low immunogenicity and other recombi-
nant proteins having this tag did not appear to have their biologic or antigenic status compromised [15]. The polyclonal rabbit anti-Pg rHtpG antibody was quite specific and sensitive, as evidenced by its ability to detect Pg rHtpG at a 1:100,000 dilution. However, a second, weak immunoreactive band on Western immunoblotting was detected when the antibody was tested against whole cell E. coli extracts. This band (approximately 60 kDa) was determined to be the E. coli GroEL protein, the prokaryotic Hsp 60 homologue (data not shown). This immunoreactivity was removed after absorption of the rabbit anti-Pg rHtpG against E. coli.

Despite the considerable homology among stress protein families across species, the Pg HtpG has limited homology to other prokaryotic Hsp90 homologues. In fact, as we reported previously, there is a 65-amino acid region within the C-terminus of the Pg HtpG that is unique among all Hsp90 homologues in both prokaryotic and eukaryotic organisms studied to date [3]. This region may prove to be a significant factor in its pathogenesis in periodontal disease. The uniqueness of Pg HtpG is supported by the absence of significant cross-reactivity with other species as shown in the immunoblot of Fig. 2B. This was anticipated given the lack of homology with other HtpG homologues [3].

The HtpG protein does not appear to contribute significantly to the growth of the organism. This is characteristic of htpg deletion mutants constructed in other species. The E. coli htpG mutant was found to have no significant changes in growth characteristics [8]. This was also true for studies examining the htpg deletion mutant in A. actinomyceseum. [16].

Since our earlier clinical studies suggested that the presence of anti-human Hsp90 serum antibodies was protective, we hypothesized that the Pg HtpG might play a role in adherence or invasion into cells and tissues, as has been proposed for other surface constituents of Pg such as fimbriae and hemagglutinins [17], and for stress proteins in other species. For example, Hsp66 was implicated in the adherence of Salmonella typhimurium to the intestinal mucus [18]. Therefore, it was logical to compare the effectiveness of adherence and invasion of the Pg W83 ΔhtpG and Pg W83 wild-type. However, we observed no differences in adherence and invasion between Pg W83 wild-type and Pg W83 ΔhtpG. Interestingly, in an earlier investigation we determined that heat-stressed upregulation of the Pg HtpG decreased adherence of wild-type Pg strains to KB cells by 15–82% (unpublished data). However, these experiments are difficult to interpret since numerous proteins are upregulated during heat stress [12].

Therefore, we conclude that HtpG does not have a significant role in the adherence and invasion processes of Pg into oral tissues as part of the pathogenesis of the organism. This, however, questions the true role of HtpG in virulence and why anti-Hsp90 antibodies appear to be protective in periodontal disease, suggesting that HtpG may be involved in either (1) critical receptor interactions pre-invasion, or (2) host cellular mechanisms important during post adherence/invasion. Studies are currently in progress in our lab to address these issues.

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References


