The role of the RgpA–Kgp proteinase–adhesin complexes in the adherence of *Porphyromonas gingivalis* to fibroblasts


Correspondence
Eric C. Reynolds
e.reynolds@unimelb.edu.au

1 Cooperative Research Centre for Oral Health Science, School of Dental Science, Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, 720 Swanston Street, Victoria 3010, Australia
2 Cooperative Research Centre for Chronic Inflammatory Diseases, Department of Medicine, The University of Melbourne, Melbourne, Victoria, Australia

*Porphyromonas gingivalis* strains W50 and ATCC 33277 were shown to bind to cultured human fibroblast (MRC-5) cells using flow cytometry. As the concentration of *P. gingivalis* strain W50 cells was increased relative to the concentration of MRC-5 cells, the number of W50 cells bound per MRC-5 cell increased, as did the percentage of MRC-5 cells with bacteria bound. However, this relationship was only seen for *P. gingivalis* strain ATCC 33277 at low cell concentrations: at high bacterial cell concentrations strain ATCC 33277 auto-aggregated and binding to the MRC-5 cells decreased. Strain W50 was therefore chosen to study the role of the surface proteinase–adhesin complexes (RgpA–Kgp complexes) in binding to MRC-5 cells. *P. gingivalis* W50 cells treated with an inhibitor of the RgpA–Kgp complexes exhibited reduced binding to MRC-5 cells. The purified active and proteinase-inactive RgpA–Kgp complexes competitively inhibited binding of W50 to MRC-5 cells, and isogenic mutants of W50 lacking RgpA/B and Kgp displayed reduced binding. *P. gingivalis* W50 mutant cells lacking Kgp exhibited the lowest binding to MRC-5 cells, suggesting an important role for this proteinase and its associated adhesins in binding to fibroblasts.

INTRODUCTION

Chronic periodontitis is an inflammatory disease of the supporting tissues of the teeth associated with specific bacteria, which can result in tooth loss. Among a large number of bacterial species found in the subgingival plaque biofilm, three Gram-negative bacteria, *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia*, as a consortium, have been strongly associated with clinical measures of chronic periodontitis (Socransky *et al.*, 1998; Socransky & Haffajee, 2002). *P. gingivalis* has received the most attention of the three species found in this consortium as it has been implicated as a major aetiological agent in the onset and progression of chronic periodontitis (Lamont & Jenkinson, 1998; O'Brien-Simpson *et al.*, 2003).

The pathogenicity of *P. gingivalis* has been attributed to a number of virulence factors (O'Brien-Simpson *et al.*, 2004). The Arg- and Lys-specific proteinases and their associated adhesins (RgpA–Kgp complexes) have been reported to be major virulence factors as they are able to degrade a range of host proteins and dysregulate host defence mechanisms (O'Brien-Simpson *et al.*, 2003; O'Brien-Simpson *et al.*, 2004). The RgpA–Kgp complexes also play a significant role in the adherence of *P. gingivalis* to host surfaces, including extracellular matrix proteins, haemoglobin and oral epithelial cells (Chen & Duncan, 2004; O'Brien-Simpson *et al.*, 2005; Pathirana *et al.*, 2007; Pike *et al.*, 1996).

*P. gingivalis* has been demonstrated to invade connective tissue in animal subcutaneous infection models, and the adherence of *P. gingivalis* to cells and matrix of the connecting tissue is likely to be an important step in the tissue invasion by this pathogen (O'Brien-Simpson *et al.*, 2001, 2003; van Steenbergen *et al.*, 1982). Gingival fibroblasts are the most common cell type of the periodontal connective tissue and their primary functions are in maintenance and regeneration of the periodontal tissue (Hassell, 1993; McCulloch & Bordin, 1991). A number of studies have reported that the *P. gingivalis* FimA fimbrial protein plays an important role in adherence to human gingival fibroblasts (Hamada *et al.*, 1994; Nakagawa...
et al., 2002; Ogawa et al., 1997). However, the role of the RgpA–Kgp complexes in binding to fibroblast cells has not been investigated. Thus the aim of this study was to determine the role of the RgpA and Kgp proteinase adhesins in binding of *P. gingivalis* to cultured human fibroblast cells.

**METHODS**

**Bacterial strains and growth conditions.** Lyophilized cultures of *P. gingivalis* ATCC 33277 and W50 wild-type strain and isogenic mutants rgpA (W501), kgp (W50KIA), rgpB (W50D7), rgpA rgpB (W50AB) and rgpA rgpB kgp (W50ABK) were obtained from the culture collection of the Cooperative Research Centre for Oral Health Science, The University of Melbourne, Melbourne, Victoria, Australia and have been described before (O'Brien-Simpson et al., 2001; Pathirana et al., 2007; Veith et al., 2002). Bacteria were maintained in an anaerobic chamber (MK3 anaerobic workstation; Don Whitley Scientific) at 37 °C on horse blood agar plates supplemented with 10 % (v/v) lysed horse blood. Bacterial colonies were used to inoculate brain heart infusion media containing 5 μg haemin ml⁻¹ and 0.5 μg cysteine ml⁻¹; for growth of the rgpA (W501), rgpB (W50D7) and kgp (W50KIA) *P. gingivalis* W50 isogenic mutants the medium also contained 10 μg erythromycin ml⁻¹ (O'Brien-Simpson et al., 2001).

For the rgpA rgpB (W50AB) isogenic mutant the medium also contained 1 μg tetracycline ml⁻¹ and 10 μg chloramphenicol ml⁻¹. For the rgpA rgpB kgp (W50ABK) isogenic mutant the media contained the following antibiotics: 0.5 μg tetracycline ml⁻¹, 10 μg chloramphenicol ml⁻¹ and 5 μg erythromycin ml⁻¹. *Escherichia coli* strain JM109 was grown anaerobically in Luria broth (LB) at 37 °C. Batch culture growth was monitored at 650 nm using a spectrophotometer (model 295E, Perkin Elmer). Culture purity was routinely determined as optimal as they contained 10 % (v/v) lysed horse blood. Bacterial colonies were used to inoculate the media for growth of the bacterial strain. These FITC concentrations were used in all subsequent experiments. After incubation with FITC, the bacteria were pelleted at 7000 × g for 5 min, then washed three times with PBS to remove unbound FITC and resuspended in EMEM supplemented with 1 % (v/v) L-glutamine and 25 mM HEPES. FITC labelling of *P. gingivalis* cells was found not to reduce cell viability or the Arg- or Lys-specific proteinase activity (data not shown).

**Adherence of FITC-labelled bacteria to MRC-5 cells.** MRC-5 cells were transferred to 24-well plates (Corning) and were grown to near confluence (~95 %) at a density of approximately 10⁶ cells per well using the culture conditions described above. Immediately prior to incubation with *P. gingivalis* ATCC 33277, W50, proteinase isogenic mutants or *E. coli*, the cell culture medium was removed and the MRC-5 cell monolayers were washed twice with sterile PBS. FITC-labelled bacteria were prepared as described above. Immediately prior to incubation with MRC-5 cells, the optical density of the bacterial solution was measured at 650 nm and the bacterial concentration was adjusted to 2.5 × 10⁸ bacteria ml⁻¹ by resuspending in EMEM supplemented with 1 % (v/v) glutamine and 25 mM HEPES. To determine the incubation period needed to obtain maximum adherence, 200 μl aliquots of FITC-labelled bacteria [5 × 10⁶ bacterial cells, bacterium to fibroblast cell ratio (BCR)=500:1 (this ratio represents the ratio of the number of bacterial cells to the number of MRC-5 cells)] were added to wells containing MRC-5 cell monolayers. The cell culture plates were then centrifuged at 400 g for 5 min at room temperature and incubated for 15, 30, 60 and 90 min at 37 °C in the anaerobic chamber (Don Whitley Scientific). A 90 min incubation period was sufficient to obtain maximum adherence and was used in all subsequent experiments. To compare the ability of *P. gingivalis* strains W50 and ATCC 33277 to bind to MRC-5 cells, FITC-labelled *P. gingivalis* W50 and ATCC 33277 were incubated with MRC-5 cell monolayers at BCRs of 10:1, 50:1, 100:1, 500:1 and 1000:1 for 90 min at 37 °C. To determine the role of Arg-specific and Lys-specific proteinases and their associated adhesins in adherence to MRC-5 cells, *P. gingivalis* W50 rgpA, rgpB, kgp, rgpA rgpB or rgpA rgpB kgp isogenic mutants were incubated with MRC-5 cell monolayers at a BCR of 500:1 for 90 min at 37 °C. Following incubation, the supernatants containing unbound bacteria and detached MRC-5 cells were removed from the wells and collected into 1 ml tubes. The remaining MRC-5 cells were then detached from the wells by incubating with a trypsin/EDTA mixture (300 μl per well) (JRH Biosciences) for 5 min at 37 °C. The detached MRC-5 cells were collected and pooled with the corresponding collected supernatants, which contained the previously detached MRC-5 cells and the unbound bacteria. The MRC-5 cells were then centrifuged at 400 g for 5 min at room temperature and washed twice in PBS to remove the unbound bacteria. After washing, the MRC-5 cells were resuspended in 300 μl PBS and 5 μl of either phycocerythrin (PE)-conjugated anti-CD29 IgG antibodies (CD29-PE) (BD Pharmingen) or the isotype-matched antibody control (PE mouse IgG1, BD Pharmingen) and incubated for 30 min on ice. Following incubation, the MRC-5 cells were centrifuged at 800 g, for 5 min at room temperature, washed twice in PBS and resuspended in 500 μl fixative solution (PBS containing 1 % (v/v) formalin) and stored at 4 °C until analysed by flow cytometry within 24 h.

**Competitive inhibition of *P. gingivalis* adherence to MRC-5 cells by the RgpA–Kgp proteinase–adhesin complexes.** The ability of both catalytically active and inactive RgpA–Kgp complexes to inhibit FITC-labelled *P. gingivalis* W50 adherence to MRC-5 cells was analysed. The RgpA–Kgp proteinase–adhesin complexes were purified from Triton X-114 extracts of *P. gingivalis* W50 using the method described previously (Pathirana et al., 2006). The Arg- and Lys-specific proteinase activity of the purified complexes was inactivated by incubating with 5 mM of the proteinase inhibitor N'-tosyl-lysine chloromethyl ketone (TLCK) (Sigma) for 30 min at 37 °C. The excess TLCK was removed by using a PD-10 desalting column, equilibrated with TC50 buffer (50 mM NaCl, 5 mM CaCl₂, 5 mM Tris/HCl, pH 7.4) and the proteinase-inhibited RgpA–Kgp...
complexes were eluted with TC50 buffer according to the manufacturer’s instructions (Amersham Pharmacia Biotech). FITC-labelled 

*P. gingivalis* W50 cells (5 x 10^6 cells) were mixed with 3.12, 6.25 or 12.5 mg ml^{-1} of catalytically active or inactive RgpA–Kgp complexes and immediately added to wells containing MRC-5 cell monolayers at a BCR of 500:1 and the adherence assay was performed as described above.

**Treatment of *P. gingivalis* W50 cells with TLCK.** The adherence of TLCK-treated *P. gingivalis* W50 whole cells to MRC-5 cell monolayers was also studied. FITC-labelled *P. gingivalis* W50 cells were incubated with 5 mM TLCK for 30 min at 37 °C in the anaerobic chamber with consistent mixing. After the incubation, the bacteria were centrifuged at 7000 g for 5 min at room temperature and the excess TLCK was removed by washing the bacteria twice in PBS (7000 g, 5 min, room temperature). The bacterial pellet was resuspended in EMEM containing 1% (v/v) glutamine and 25 mM HEPES at a concentration of 2.5 x 10^9 *P. gingivalis* ml^{-1} and the adherence assay was performed as described above.

**Fluorescence microscopy.** The adherence of *P. gingivalis* strains W50 and ATCC 33277 to MRC-5 cells was visualized using a fluorescence microscope (model L5, Leica). A 10 μl drop of the sample was added onto mounting fluid (Aqua PolyMount, PolySciences) placed on a glass slide and overlaid with a coverslip. Adherence of FITC-labelled bacteria was examined using a blue excitation filter (excitation 450/64 nm, emission 550/54 nm) under oil immersion (× 100 magnification).

**Flow cytometric analysis of FITC-labelled *P. gingivalis* and *E. coli* adherence to MRC-5 cells.** The adherence of FITC-labelled *P. gingivalis* to MRC-5 cells stained with CD29-PE was analysed using a FACSCaliber flow cytometer (Becton Dickinson) equipped with an argon laser operating at an excitation wavelength of 488/610 nm. The fluorescence from PE was measured through a 575 nm filter (FL2) and the green emission of FITC was measured with a 525 nm filter (FL1). The multiparametric data were analysed by CellQuest software (Becton Dickinson). Forward and side scatter properties were used to acquire a total of 10 000 MRC-5 cells and to gate out the cell debris. MRC-5 cells were then specifically identified by gating for PE fluorescence (FL2). MRC-5 cells that had FITC-labelled *P. gingivalis* attached were identified by gating on the FITC fluorescence (FL1) within the gated PE region. All measurements were done in duplicate and for quantification of FITC fluorescence, mean fluorescence intensity (MFI) values were used.

**Statistical analysis.** The FITC MFI values of MRC-5 cells incubated with FITC-labelled *P. gingivalis* W50 wild-type or isogenic mutants lacking either *rgpA*, *rgpB*, *kgp*, *rgpA rgpB*, or *rgpA rgpB kgp* gene products were analysed using a one-way classification ANOVA and SPSS software. Regression analysis of the percentage of MRC-5 cells with *P. gingivalis* strains W50 and ATCC 33277 attached and inhibition of *P. gingivalis* W50 binding to MRC-5 cells by RgpA–Kgp complex was performed using SPSS software. Effect sizes, represented as Cohen’s *d*, were calculated using the effect size calculator provided online by Evidence-Based Education UK website at http://www.ebcentre.org/enderpage.asp?linkid=30325016. According to Cohen (1969) a small effect size is *d* >0.2 and <0.5, moderate *d* >0.5 and <0.8 and large *d* >0.8.

**RESULTS**

**Flow cytometric analysis of bacterial binding to MRC-5 cells**

Initial experiments using fluorescence microscopy showed that *P. gingivalis* W50 bound to MRC-5 cells as either single cells or very small aggregates (Fig. 1A, C), whereas *P. gingivalis* strain ATCC 33277 bound to MRC-5 cells predominantly as large cell aggregates (Fig. 1B, D). FITC-labelled *E. coli* were found not to bind to MRC-5 cells (data not shown).

The adherence of FITC-labelled *P. gingivalis* to MRC-5 cells was analysed by flow cytometry (Fig. 2). Cell debris, unbound bacteria and cell clumps were excluded from analysis by forward and side scatter gating (gate 1, G1, Fig. 2A). MRC-5 cells labelled with CD29-PE antibody were then identified by gating on the PE fluorescence (peak

---

**Fig. 1.** (A, B) Fluorescence microscopic images of MRC-5 cells with FITC-labelled *P. gingivalis* W50 (A) and FITC-labelled *P. gingivalis* ATCC 33277 (B) attached. (C, D) Light microscopic images of the same MRC-5 cell bound with *P. gingivalis* W50 (C) and *P. gingivalis* ATCC 33277 (D). Magnification of all figures is ×50.
G2, Fig. 2B) that was above the PE fluorescence obtained from MRC-5 cells stained with the isotype-matched IgG-PE control (peak 2, Fig. 2B). It was found that 99.0% of the MRC-5 cell population was labelled with CD29-PE antibody (peak 1, Fig. 2B) and 1.0% of MRC-5 cells were positive for IgG-PE control (peak 2, Fig. 2B). Only CD29-PE-positive MRC-5 cells were used to determine bacterial adherence, in terms of both the percentage of MRC-5 cells with FITC-labelled P. gingivalis attached and the relative amount of P. gingivalis attached as determined by the MFI of FITC fluorescence. MRC-5 cells (CD29-positive) with FITC-labelled P. gingivalis attached were identified by gating on FITC fluorescence (G3, Fig. 2C) above the limit of autofluorescence of CD29-PE-positive MRC-5 cells incubated with non-FITC-labelled P. gingivalis (peak 3, Fig. 2C) and FITC fluorescence of non-adherent FITC-labelled E. coli co-eluting with CD29-PE-positive MRC-5 cells (peak 4, Fig. 2C). Typically, it was found that 93.1% of CD29-PE-positive MRC-5 cells had FITC-labelled P. gingivalis attached (peak 5, Fig. 2C) and that there was 6.2% co-elution of CD29-PE-positive MRC-5 cells with the non-adherent FITC-labelled E. coli cells. These flow cytometric controls and settings were used for all subsequent experiments to determine P. gingivalis adherence to MRC-5 cells.

**Adherence of P. gingivalis strains W50 and ATCC 33277 to MRC-5 cells**

The adherence of P. gingivalis strain W50 to MRC-5 cells was compared to that of strain ATCC 33277 by flow cytometry. Fig. 3(A) shows that there is an increase in FITC MFI of MRC-5 cells incubated with increasing numbers of P. gingivalis W50. Regression analysis demonstrated that there was a significant (P<0.0001) positive linear relationship (R²=0.999) between FITC MFI values and the bacterium fibroblast cell ratio (BCR) (Fig. 3A). Furthermore, the percentage of MRC-5 cells with P. gingivalis W50 attached increased with a corresponding increase in the numbers of P. gingivalis W50 incubated with MRC-5 cells (Fig. 3A). The percentage of MRC-5 cells with P. gingivalis W50 attached reached a maximum of 88.0%±1.4% at a BCR of 500:1 and remained constant at a BCR of 1000:1. Regression analysis showed that the binding of P. gingivalis W50 to MRC-5 cells exhibited a significant linear relationship with the BCR (R²=0.882, P<0.0546).

When MRC-5 cells were incubated with P. gingivalis ATCC 33277, it was again found that there was an increase in FITC MFI values of MRC-5 cells with a corresponding increase in BCR, and regression analysis showed that this was a significant (P<0.0248) positive linear relationship (R²=0.854) relationship (Fig. 3B). The percentage of MRC-5 cells with P. gingivalis ATCC 33277 attached was found to increase with BCR and reached a maximum of 82.0%±12.0% at a BCR of 100:1; however, at the higher BCR of 1000:1 this decreased to only 26.1%±0.7% (Fig. 3B). Regression
analysis defined this binding pattern as a quadratic (inverted U) relationship ($R^2=0.856$, $P<0.0859$).

### Adherence of *P. gingivalis* W50 and *rgpA*, *rgpB*, *kgp*, *rgpA rgpB* and *rgpA rgpB kgp* isogenic mutants to MRC-5 cells

The ability of *P. gingivalis* W50 wild-type to adhere to MRC-5 cells was compared to that of a series of *P. gingivalis* W50 isogenic mutants, *rgpA*, *rgpB*, *kgp*, *rgpA rgpB* and *rgpA rgpB kgp* using flow cytometry (Fig. 4). The percentage of MRC-5 cells with *kgp* (77.3 ± 1.56%) and *rgpA rgpB kgp* (72.2 ± 1.8%) isogenic mutants attached were shown to be significantly ($P<0.01$) lower compared to that of *P. gingivalis* W50 wild-type (93.8 ± 0.5%).

Furthermore, the FITC MFI values were also significantly ($P<0.01$) lower in MRC-5 cells incubated with either *kgp* (118.0 ± 8.5) or *rgpA rgpB kgp* (102.0 ± 5.6) isogenic mutants compared with that of *P. gingivalis* W50 wild-type (325.0 ± 20.5). The percentage of MRC-5 cells with *rgpA* (90.8 ± 0.6), *rgpA rgpB* (92.05 ± 0.5) and *rgpB* (95.9 ± 0.8) isogenic mutants attached was not found to be significantly different from the percentage with *P. gingivalis* W50 wild-type. However, the FITC MFI values were significantly ($P<0.05$) lower in MRC-5 cells incubated with either *rgpA* (204.0 ± 20.5), *rgpA rgpB* (182.8 ± 62.5) or *rgpB* (194.3 ± 16.5) isogenic mutants compared with that of *P. gingivalis* W50 wild-type.

### Role of RgpA–Kgp proteinase–adhesin complexes in adherence of *P. gingivalis* W50 to MRC-5 cells

The contribution of Arg- and Lys-specific proteinase activity to binding of *P. gingivalis* strain W50 to MRC-5 cells was investigated by pre-treatment of *P. gingivalis* W50 whole cells with the trypsin proteinase inhibitor TLCK. The FITC MFI value of bacteria bound to MRC-5 cells incubated with TLCK-treated *P. gingivalis* W50 was significantly ($P<0.01$) lower compared with MRC-5 cells incubated with non-TLCK-treated *P. gingivalis* W50 cells.
(Fig. 5A). No significant difference was observed between proteinase-active and inactive P. gingivalis with regard to the percentage of MRC-5 cells with bacteria attached (data not shown).

To further investigate the role of the RgpA–Kgp complexes in P. gingivalis binding to MRC-5 cells, assays were performed where MRC-5 cell monolayers were incubated with P. gingivalis W50 (BCR 500:1) in the presence of increasing concentrations of either proteinase-active or proteinase-inactive (TLCK-treated) RgpA–Kgp complexes (Fig. 5B). Incubation with proteinase-active and -inactive RgpA–Kgp complexes inhibited P. gingivalis binding to MRC-5 cells in a dose-dependent manner and concentrations of 6.25 µg ml⁻¹ and above significantly (P<0.01) reduced the FITC MFI values of bacteria bound to MRC-5 cells. The proteinase-active RgpA–Kgp complexes tended to be more effective at inhibiting binding of P. gingivalis W50 to MRC-5 cells as suggested by the higher effect sizes compared with proteinase-inactive RgpA–Kgp complexes. Incubation with either proteinase-active or -inactive RgpA–Kgp complexes did not significantly reduce the percentage of MRC-5 cells with P. gingivalis W50 attached (data not shown).

**DISCUSSION**

In this study, P. gingivalis strains W50 and ATCC 33277 were shown to bind to MRC-5 fibroblast cells by fluorescence microscopy and flow cytometric analysis. However, strain ATCC 33277 displayed a complex pattern of binding to fibroblast cells which was attributed to auto-aggregation at high bacterial cell numbers; hence strain W50 was chosen to study the role of the RgpA–Kgp proteinase–adhesin complexes in fibroblast binding. The RgpA–Kgp complexes are major virulence factors of P. gingivalis and inhibition of Arg- and Lys-specific proteinase activity of whole cells by using a proteinase inhibitor (TLCK) significantly reduced the adherence of P. gingivalis W50 to MRC-5 cells. This suggests that the Arg- and Lys-specific proteinase activity may play a role in adherence of P. gingivalis W50 to MRC-5 cells. Furthermore, both active and inactive (TLCK-treated) purified RgpA–Kgp complexes competitively inhibited adherence of P. gingivalis W50 to MRC-5 cells; however, the proteinase-inactive RgpA–Kgp complexes tended to display a smaller inhibitory effect. The fact that the TLCK-inactivated RgpA–Kgp complexes still competitively inhibited binding of P. gingivalis W50 to fibroblast cells is consistent with the adhesin domains playing a role in binding; this extends the earlier work of Chen & Duncan (2004), who showed that the adhesin domains were important for binding to epithelial cells. Considered together, these results suggest that the Arg- and/or Lys-specific proteinases may mediate adherence through cleaving hidden or latent surface receptors on MRC-5 cells to which the RgpA–Kgp complexes then adhere through their adhesin domains. This mechanism is similar to that previously described for P. gingivalis W50 adherence to fibronectin (Kontani et al., 1997) and epithelial cells (Pathirana et al., 2007).

The role of Arg- and Lys-specific proteinases and their associated adhesins in the adherence of P. gingivalis W50 to MRC-5 cells was further studied using isogenic mutants lacking the rgpA, rgpB and kgp gene products. A significant reduction in the FITC MFI values was observed for the rgpA, rgpB, and kgp gene products. A significant reduction in the FITC MFI values was observed for the rgpA and kgp gene products. A significant reduction in the FITC MFI values was observed for the rgpB and kgp gene products. A significant reduction in the FITC MFI values was observed for the rgpA and kgp gene products. A significant reduction in the FITC MFI values was observed for the rgpB and kgp gene products. A significant reduction in the FITC MFI values was observed for the rgpA and kgp gene products.

The role of Arg- and Lys-specific proteinases and their associated adhesins in the adherence of P. gingivalis W50 to MRC-5 cells was further studied using isogenic mutants lacking the rgpA, rgpB and kgp gene products. A significant reduction in the FITC MFI values was observed for the rgpA, rgpB, and kgp gene products.
Previous studies have reported that *P. gingivalis* fimbriae play a significant role in mediating the adherence of strains ATCC 33277 and 381 to gingival fibroblast cells (Hamada et al., 1994; Kontani et al., 1996; Ogawa et al., 1997). Furthermore, Kontani et al. (1996) have suggested synergism between the fimbriae and the Arg-specific proteinases in mediating the adherence of *P. gingivalis* ATCC 33277-like strains to fibroblast cells. However, as *P. gingivalis* W50 is sparsely fimbriated, adherence of this strain to fibroblast cells may not be mediated by fimbriae and the results of the current study suggest that the proteinases and their associated adhesin domains, especially Kgp, also have a role in adherence.

*P. gingivalis* strains W50 and ATCC 33277 were found to have significantly different binding patterns to MRC-5 cells. Initial experiments using fluorescence microscopy showed that *P. gingivalis* W50 bound to MRC-5 cells as single cells or very small aggregates whereas strain ATCC 33277 bound as large cell aggregates. Flow cytometric and regression analysis revealed a logarithmic relationship for binding of *P. gingivalis* W50 to MRC-5 cells whereas strain ATCC 33277 was found to have a quadratic (inverted U) binding relationship. This quadratic binding relationship for *P. gingivalis* ATCC 33277 may be explained by the auto-aggregation of cells at high concentrations, in preference to binding to host cells. In contrast, the logarithmic relationship observed for *P. gingivalis* W50 may be a result of binding to MRC-5 cells as single cells or small clumps of cells. This binding pattern, which was also observed for binding to KB oral epithelial cells (Pathirana et al., 2007), may help explain the differences in the invasive potential in the animal subcutaneous lesion model of *P. gingivalis* W50 (invasive) and ATCC 33277 (non-invasive) strains. The *P. gingivalis* strain W50 has been described as being more invasive in animal subcutaneous lesion models than strains ATCC 33277 and 381, as W50 produces spreading, ulcerated primary lesions with secondary lesions while ATCC 33277 and 381, as W50 produces spreading, ulcerated primary lesions with secondary lesions while ATCC 33277 was found to have a quadratic (inverted U) binding relationship. This quadratic binding relationship for *P. gingivalis* ATCC 33277 may be explained by the auto-aggregation of cells at high concentrations, in preference to binding to host cells. In contrast, the logarithmic relationship observed for *P. gingivalis* W50 may be a result of binding to MRC-5 cells as single cells or small clumps of cells. This binding pattern, which was also observed for binding to KB oral epithelial cells (Pathirana et al., 2007), may help explain the differences in the invasive potential in the animal subcutaneous lesion model of *P. gingivalis* W50 (invasive) and ATCC 33277 (non-invasive) strains. The *P. gingivalis* strain W50 has been described as being more invasive in animal subcutaneous lesion models than strains ATCC 33277 and 381, as W50 produces spreading, ulcerated primary lesions with secondary lesions while ATCC 33277 produces a localized abscess (Neiders et al., 1989; van Steenbergen et al., 1982). For *P. gingivalis* ATCC 33277, formation of large cell aggregates may impede its ability to invade connective tissue. However, as *P. gingivalis* W50 has a low auto-aggregation potential, this characteristic may facilitate invasion.

*P. gingivalis* ATCC 33277-like strains have been reported previously to auto-aggregate compared with W50-like strains (Kuramitsu et al., 1997; Pathirana et al., 2007). Further to this, auto-aggregation has also been reported to significantly affect the virulence of other pathogenic bacteria (Bieber et al., 1998; Sheikh et al., 2002). In enteroaggregative *E. coli* (EAEC), a group of extracellular enzymes designated ‘dispersins’ are reported to counteract bacterial auto-aggregation, and in enteropathogenic *E. coli* (EPEC) the bundle-forming pili (BFP) facilitate both bacterial aggregation and dispersal (Knutton et al., 1999; Sheikh et al., 2002). Mutant strains of EAEC and EPEC lacking dispersin and BFP have been reported to autoaggregate, forming large cell aggregates that are less invasive and less virulent compared with the wild-types (Bieber et al., 1998; Sheikh et al., 2002).

In summary, the present study shows that *P. gingivalis* strain W50 binds to MRC-5 fibroblast cells and that the Arg- and Lys-specific proteinases and their associated adhesins, especially Kgp, play a role in this process. *P. gingivalis* strains W50 and ATCC 33277 were found to have different binding patterns to MRC-5 cells, which was attributed to auto-aggregation of strain 33277 at high cell concentrations.

**ACKNOWLEDGEMENTS**

The authors would like to thank Ms Narelle Skinner for assistance with fluorescence microscopy. This project was supported by the National Health and Medical Research Council (Project Grant 454475) and the National Institutes of Health (Grant 1 R01DE14198-01).

**REFERENCES**


Edited by: P. E. Kolenbrander