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

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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.

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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 Steenberg *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

Abbreviations: BCR, bacterium to fibroblast cell ratio; MFI, mean fluorescence intensity; PE, phycoerythrin; TLCK, *N*-(*p*-tosyl)-lysine chloromethyl ketone.

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* (W50K1A), *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* (W50K1A) *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 checked by Gram stain and by colony morphology.

Human fibroblast (MRC-5) cell culture. The immortalized human fibroblast cell line MRC-5 (CSL Ltd) was maintained as frozen stocks and cultured in Earle's minimum essential medium (EMEM) supplemented with 25 mM L-glutamine, 10% (v/v) heat-inactivated fetal calf serum, 100 IU penicillin/streptomycin ml⁻¹, and 30 µg gentamicin ml⁻¹ (JRH Biosciences) in a 5% CO₂ atmosphere at 37 °C. Cell passage (1:3 split) was performed twice a week, and the fibroblast cells used in experiments were never older than 12 passages.

FITC labelling of *P. gingivalis* and *E. coli*. *P. gingivalis* strains ATCC 33277, W50 wild-type and *P. gingivalis* W50 proteinase isogenic mutants *rgpA*, *kgp*, *rgpB*, *rgpA rgpB* and *rgpA rgpB kgp* and *E. coli* strain JM109 were grown to mid-exponential phase as described above. Bacterial concentrations were determined spectrophotometrically according to a specific standard curve and confirmed retrospectively by counting viable cell colonies plated on blood agar for *P. gingivalis* and on LB plates for *E. coli*. The bacteria were harvested by centrifugation at 7000 g for 20 min at 4 °C and washed once in PBS, pH 7.4. In order to select an optimal concentration for bacterial labelling, *P. gingivalis* strains ATCC 33277 and W50 and *E. coli* (2.5 × 10⁹ ml⁻¹) were resuspended in 0.5 M NaHCO₃, pH 8.0, containing different concentrations of FITC (Invitrogen) and were incubated for 30 min at 37 °C in an anaerobic chamber with constant stirring. A FITC concentration of 0.15 mg ml⁻¹ for *P. gingivalis* W50 wild-type and the proteinase mutants and *E. coli* and 0.015 mg ml⁻¹ for *P. gingivalis* ATCC 33277 was determined as optimal as they yielded homogeneous labelling and a suitable staining intensity (i.e. no significant difference in fluorescence intensity between each 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 × 10⁹ 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 × 10⁸ 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 phycoerythrin (PE)-conjugated anti-CD29 IgG antibodies (CD29-PE) (BD Pharmingen) or the isotype-matched antibody control (PE mouse IgG₁K, 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*^ε-p-tosyl-L-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 (5×10^8 cells) were mixed with 3.12, 6.25 or $12.5 \mu\text{g ml}^{-1}$ 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 2.5×10^9 *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 ($\times 100$ 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*, *kpg*, *rgpA rgpB*, or *rgpA rgpB kpg* 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.cem-centre.org/renderpage.asp?linkid=30325016>. According to Cohen (1969) a small effect size is $d \geq 0.2$ and < 0.5 , moderate $d \geq 0.5$ and < 0.8 and large $d \geq 0.8$.

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 $\times 50$.

Fig. 2. Development of a flow cytometric method for analysis of bacterial adherence to MRC-5 cells. FITC-labelled bacteria were incubated with MRC-5 cells for 90 min at a BCR of 500:1, harvested, washed and stained with CD29-PE. (A) Live MRC-5 cells were gated according to forward and side scatter properties (G1). (B) MRC-5 cells were stained with negative isotypic IgG1-PE control (peak 2) or CD29-PE (peak 1). MRC-5 cells with bacteria attached were analysed by gating on the FITC fluorescence (G3) within the gated PE region (G2). (C) Peak 3 represents autofluorescence of MRC-5 cells incubated with non-FITC-labelled *P. gingivalis* W50. Peak 4 represents FITC fluorescence of MRC-5 cells with *E. coli* strain JM109, a non-adherent Gram-negative bacterial control. Peak 5 represents FITC fluorescence of MRC-5 cells with *P. gingivalis* W50 attached. A total of 10 000 CD29-PE positive events were obtained and experiments were performed in duplicate; representative results are shown.

1, 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 ($R^2 = 0.999$) 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 $88.0 \% \pm 1.4 \%$ 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 ($R^2 = 0.882$, $P < 0.0546$).

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 ($R^2 = 0.854$) 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 $82.0 \% \pm 12.0 \%$ at a BCR of 100:1; however, at the higher BCR of 1000:1 this decreased to only $26.1 \% \pm 0.7 \%$ (Fig. 3B). Regression

Fig. 3. Adherence of FITC-labelled *P. gingivalis* to MRC-5 cell monolayers. Confluent MRC-5 cell monolayers were incubated with *P. gingivalis* W50 (A) and *P. gingivalis* ATCC 33277 (B), at BCRs of 10:1, 50:1, 100:1, 500:1 and 1000:1 for 90 min at 37 °C and adherence was analysed by flow cytometry. The results are presented as percentage of MRC-5 cells with attached *P. gingivalis* (black bars) and the FITC fluorescence emitted from the attached *P. gingivalis* as indicated by the MFI $\pm$ SD (white bars). A total of 10 000 CD29-PE positive events were obtained and experiments were performed in duplicate.

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* ($77.3\% \pm 1.56\%$) and *rgpA rgpB kgp* ($72.2\% \pm 1.8\%$) isogenic mutants attached were shown to be significantly ($P<0.01$) lower compared to that of *P. gingivalis* W50 wild-type ($93.8\% \pm 0.5\%$).

Fig. 4. Adherence of *P. gingivalis* W50 wild-type and *rgpA*, *rgpB*, *kgp*, *rgpA rgpB* and *rgpA rgpB kgp* isogenic mutants to MRC-5 cell monolayers. MRC-5 cell monolayers were incubated with the various strains at a BCR of 500:1 and adherence was measured by flow cytometry. Groups significantly different from wild-type *P. gingivalis* are marked with asterisks (*, $P<0.01$; **, $P<0.05$). The results are presented as percentage of MRC-5 cells with attached *P. gingivalis* (black bars) and the FITC fluorescence emitted from the attached *P. gingivalis* as indicated by the MFI $\pm$ SD (white bars). A total of 10 000 CD29-PE positive events were obtained and experiments were performed in duplicate.

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 $\mu\text{g ml}^{-1}$ 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*, *rgpA rgpB* ($P<0.05$) and *kgp* and *rgpA rgpB kgp* ($P<0.01$) isogenic mutants, suggesting that Kgp, RgpB and RgpA play a role in adherence of *P. gingivalis* W50 to MRC-5 cells. In addition, for the *kgp* and *rgpA rgpB kgp* isogenic mutants, the percentage of MRC-5 cells with *P. gingivalis* W50 attached was also found to be significantly lower ($P<0.01$) than that of *P. gingivalis* W50 wild-type. However, no significant difference was observed between *P. gingivalis* W50 wild-type and the *rgpA*, *rgpB* and *rgpA rgpB* isogenic mutants with regard to the percentage of fibroblast cells with *P. gingivalis* attached. These results suggest that Kgp plays a greater role in mediating adherence to fibroblast cells compared with either RgpB or RgpA.

Fig. 5. Inhibition of binding of *P. gingivalis* W50 to MRC-5 cells. (A) Adherence of proteinase-active (black bars) and inactive (white bars) *P. gingivalis* W50 (BCR 500:1) to MRC-5 cells as measured by flow cytometry. *, Group indicated significantly ($P<0.01$) different from control group (proteinase-active *P. gingivalis* W50). (B) Inhibition of *P. gingivalis* W50 adherence to MRC-5 cells by the RgpA–Kgp complexes. Confluent MRC-5 cell monolayers were incubated with FITC-labelled *P. gingivalis* W50 (BCR 500:1) in the presence of 3.12, 6.25 or 12.5 $\mu\text{g ml}^{-1}$ of either TLCK-treated (white bars) or non-TLCK-treated (black bars) RgpA–Kgp complexes. *, Groups indicated significantly different ($P<0.01$) from the control group not incubated with RgpA–Kgp complexes. All results are expressed as FITC-fluorescence as indicated by the MFI $\pm$ SD of duplicate samples. A total of 10 000 CD29-PE positive events were obtained for each experiment.

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 produces a localized abscess (Neiders *et al.*, 1989; van Steenberg *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 auto-aggregate, 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.

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