Regulation of RANKL and OPG gene expression in human gingival fibroblasts and periodontal ligament cells by *Porphyromonas gingivalis*: A putative role of the Arg-gingipains

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Abstract

*Porphyromonas gingivalis* is highly implicated in the pathogenesis of periodontitis, which is characterized by the destruction of periodontal connective tissues and the supporting alveolar bone. Receptor Activator of NF-\(\kappa\)B Ligand (RANKL) stimulates bone resorption, whereas osteoprotegerin (OPG) blocks its action, and this bi-molecular system is implicated in periodontitis. The aim of this work was (a) to investigate the regulation of RANKL and OPG gene expression in human periodontal ligament (PDL) cells and gingival fibroblasts (GF), in response to *P. gingivalis* culture supernatants, by quantitative real-time PCR and (b) to attempt to identify putative virulence factors involved in this process. The results indicated that *P. gingivalis* induced RANKL and reduced OPG mRNA expression by the studied cells, resulting in an increased RANKL/OPG expression ratio. Heat-inactivation of *P. gingivalis* resulted in significant reduction of RANKL mRNA expression. A Lys-gingipain mutant strain did not affect, whereas an Arg-gingipain mutant strain further enhanced RANKL mRNA expression, compared to their parental wild-type strain. In conclusion, *P. gingivalis* up-regulates the RANKL/OPG expression ratio in GF and PDL cells, denoting an enhanced osteoclastogenic potential by the cells. The component mainly responsible for RANKL induction appears to be proteinaceous, and it may be regulated by the Arg-gingipains.

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1. Introduction

Periodontitis is an infectious disease that leads to the destruction of the tooth-supporting tissues, including the periodontal ligament (PDL), the gingival connective tissue, and the alveolar bone. The PDL is the connective tissue mediating the functional attachment of teeth to alveolar bone, whereas the gingival connective tissue is lining the alveolar bone, protecting it from external stimuli. Although the resident cell populations of these tissues, namely gingival fibroblasts (GF) and PDL cells, may exhibit distinct phenotypic characteristics [1,2], they both establish a dynamic balance between tissue formation and degradation at the tooth–bone interface. Local inflammatory processes could cause a shift in the host response balances, and periodontitis may eventually occur [3].

*Porphyromonas gingivalis* is Gram-negative black-pigmented anaerobe, considered to have a protagonistic role in chronic periodontitis [4]. Putative virulence factors of this pathogen include its lipopolysaccharide (LPS) [5] and Arg- and Lys-specific cysteine proteinases, or gingipains [6], which are agents capable of perturbing local immunity and deregulating the host’s inflammatory response. *P. gingivalis* also possesses a number of surface adhesion molecules, such as fimbriae, which enable it to adhere to extracellular matrix components, as well as the cell membrane of eukaryotic cells, including GF and epithelial...
2. Results

2.1. Cytotoxic effects of P. gingivalis

In order to define the concentration and time-range to be used, the putative toxicity of P. gingivalis culture supernatants on GF and PDL cells was firstly investigated. After 6 h of bacterial challenge, P. gingivalis protein concentrations ≤ 50 µg/ml did not elicit strong cytotoxicity, whereas higher concentrations resulted in cell detachment. Concomitantly, an increase was observed in extracellularly released lactate dehydrogenase (LDH) with P. gingivalis concentrations greater than 50 µg/ml, indicative of increased cell death. (Fig. 1). After 24 h of bacterial challenge, almost no viable cells were detected in response to 100 µg/ml P. gingivalis protein, whereas a slight increase in cytotoxicity was also observed with lower concentrations (Fig. 1). Subsequently, sub-toxic conditions were selected for further experiments on gene expression analysis, including P. gingivalis concentrations up to 50 µg/ml.

2.2. Effect of P. gingivalis on RANKL and OPG mRNA expression

The effect of P. gingivalis on RANKL and OPG mRNA expression was next evaluated by quantitative real-time PCR. Neither GF, nor PDL cells expressed RANKL under physiological conditions. However, after 6 h of bacterial challenge, both GF and PDL cells were induced to express RANKL in concentration-dependent manner (Fig. 2). On the contrary, OPG was constitutively expressed in both cell types, and a 6 h challenge with P. gingivalis decreased its expression.
expression (Fig. 3). However, this decrease in OPG expression at 6 h proved to be significant only in PDL cells, but not in GF, with the highest *P. gingivalis* protein concentration used (50 µg/ml). The changes observed in RANKL and OPG expression after 6 h of bacterial challenge resulted in an increased RANKL/OPG expression ratio by both cell types (Fig. 4). This increase was significant with concentrations equal or higher than 6.25 µg/ml *P. gingivalis* protein.

The expressions of RANKL and OPG in response to *P. gingivalis* were further investigated after 24 h of challenge. Between 6 and 24 h of bacterial challenge, RANKL expression was further increased by 2.35-fold in GF, but it decreased by four-fold in PDL cells (Fig. 5A). On the contrary, over the same interval, OPG was decreased by 40-fold in GF, but increased by 16-fold in PDL cells (Fig. 5B). As a consequence of these changes in RANKL and OPG expression over 24 h, their relative RANKL/OPG ratio was differentially regulated in GF and PDL cells. After the initial 6 h peak in PDL cells, this was decreased by 100-fold. However, after the initial induction at 6 h in GF, it was further increased by 75-fold in GF (Fig. 5C).

### 2.3. Involvement of Lys- and Arg-gingipains

The next step was to attempt to identify the nature of *P. gingivalis* virulence factor involved in the observed regulations of RANKL and OPG expression. Firstly, to determine if the involved factor(s) are proteins, culture supernatant from wild-type *P. gingivalis* W50 strain was heat-treated at 70°C for 1 h, and its effects on GF were compared to those of the untreated supernatant. The results indicated that compared to untreated *P. gingivalis*, heat-treatment resulted in a four-fold reduction of RANKL expression (Fig. 6A), but did not affect OPG expression (Fig. 6B) in GF. The involvement of the Arg-X and Lys-X proteinases (gingipains) was further investigated by employing culture supernatants from corresponding *P. gingivalis* mutant strains (E8: Arg-X proteinase mutant; K1A: Lys-X proteinase mutant) and comparing their effects to those of equal concentrations of the wild-type W50 strain (50 µg/ml). There was no difference in RANKL, or OPG expression between the K1A and its parental W50 wild-type strain. However, the Arg-X proteinase-deficient *P. gingivalis* E8 strain caused a 5.8-fold greater up-regulation of RANKL expression compared to the W50 wild-type strain, but did not affect OPG expression (Figs. 6A and B).
Clinical studies have demonstrated that the RANKL/OPG ratio is increased in periodontally diseased tissues [18,20]. The present work demonstrates that P. gingivalis secreted products induce RANKL and down-regulate OPG mRNA expression in human periodontal connective tissue cells, resulting in an increased RANKL/OPG expression ratio. This may denote an enhanced capacity of the resident periodontal cells/tissues to locally stimulate osteoclastogenesis and bone resorption by pre-osteoclasts recruited in the area, via the blood microcirculation. In addition to GF and PDL cells, P. gingivalis may potentially exert these effects on T and B lymphocytes as well. These cells are considered an important source of RANKL in periodontally diseased sites [10,34,35], and in vivo studies have demonstrated their involvement in alveolar bone destruction, in response to P. gingivalis [36–38] and A. actinomycetemcomitans [39–42] infection.

The findings of the present study are in agreement with previous works demonstrating that P. gingivalis components may induce RANKL expression in PDL cells [31], osteoblasts [43–45] and cementoblasts [46]. A recent work demonstrated by semi-quantitative RT-PCR that P. gingivalis induces RANKL expression in human PDL cells after 24 h of challenge [31]. In the present work, the more sensitive quantitative real-time PCR has been employed, and RANKL expression was also investigated at the earlier time point of 6 h post-challenge. Following the initial 6 h concentration-dependent peak, RANKL expression was decreased at 24 h, while that of OPG was increased, corroborating recent findings in osteoblasts on the mRNA level [45], and cementoblasts on the protein level [46]. As a result of these regulations, P. gingivalis-challenged PDL cells exhibited a significantly higher RANKL/OPG ratio at 6 h, than at 24 h (Fig. 5C), indicating a transient increase in their osteoclastogenic potential. This may render them more pathogenic at earlier stages of periodontal infection. Alternatively, this reversed over time RANKL/OPG ratio could imply that PDL cells are adapted to have a long-term protective effect on bone resorption in response to infection, a view shared on cementoblasts and root resorption [46]. On the contrary, P. gingivalis-challenged GF demonstrated a steady enhancement in RANKL expression, and a delayed decrease in OPG expression over 24 h, resulting in a dramatic increase of the RANKL/OPG ratio. These observations may denote either that GF respond slower than PDL cells to P. gingivalis in terms of RANKL and OPG regulation, or that GF are more detrimental in establishing longer term bone resorption.

3. Discussion

Clinical studies have demonstrated that the RANKL/OPG ratio is increased in periodontally diseased tissues [18,20]. The present work demonstrates that P. gingivalis secreted products induce RANKL and down-regulate OPG mRNA expression in human periodontal connective tissue cells, resulting in an increased RANKL/OPG expression ratio. This may denote an enhanced capacity of the resident periodontal cells/tissues to locally stimulate osteoclastogenesis and bone resorption by pre-osteoclasts recruited in the area, via the blood microcirculation. In addition to GF and PDL cells, P. gingivalis may potentially exert these effects on T and B lymphocytes as well. These cells are considered an important source of RANKL in periodontally diseased sites [10,34,35], and in vivo studies have demonstrated their involvement in alveolar bone destruction, in response to P. gingivalis [36–38] and A. actinomycetemcomitans [39–42] infection.

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the capacity of

Fig. 6. Effect of Arg-X and Lys-X proteinase mutants on the regulation of RANKL and OPG expression in GF. GF cultures were challenged for 6 h with 50 μg/ml culture supernatant protein from P. gingivalis strains W50, heat-treated W50, E8 (Arg-X proteinase mutant) and K1A (Lys-X proteinase mutant). The mRNA expression levels of RANKL (A) and OPG (B) were measured by quantitative real-time PCR analysis, and were normalized against the expression levels of 18S rRNA (internal control). Bars represent mean values ± SEM from five independent experiments in one GF cell line.

Still, these differential regulations underpin the distinct phenotypic identity of these two cell types.

Whether the observed regulations of RANKL and OPG gene expression in the present system are direct effects, or mediated by other inflammatory mediators, is not clear. Previous work has indicated that after 3 days of challenge, P. gingivalis induced RANKL and reduced OPG expression in murine osteoblasts, via the mediation of prosta-
glandin E2 [43]. Nevertheless, in the present experimental system RANKL expression was already induced after 6 h of challenge with P. gingivalis, allowing for only limited time for the involvement of other inflammatory mediators.

To this extent, it has previously been shown that A. actinomycetemcomitans induces RANKL expression in human GF, independently of the classical inflammatory mediators [33].

After establishing that P. gingivalis induces changes in RANKL and OPG expression by periodontal connective tissue cells, the next step was to attempt to identify the nature of the involved virulence factors. A classical approach employed in this study was to heat-treat the bacterial supernatant, a procedure that inactivates the protein component, but not the LPS or other carbohydrates. The outcome of this treatment was a reduction in the capacity of P. gingivalis to induce RANKL in GF by 75%, although OPG was not affected. These findings are in direct agreement with a previous report demonstrating that heat-inactivated P. gingivalis cells exhibited diminished capacity to stimulate RANKL expression in murine osteoblasts by 70% [45]. This indicates that the observed RANKL induction largely accounts for a proteinaceous component(s) present in the P. gingivalis culture supernatant. However, the possibility of LPS involvement should not be excluded, at least for the remaining 25% of the stimulating capacity of P. gingivalis. To this extent, purified P. gingivalis LPS was sufficient to induce RANKL expression in murine cementoblasts [46]. The levels of RANKL induction may as well vary depending on the cell type and the experimental setup employed in each study.

One of the previous studies hinted that P. gingivalis gingipains may be involved in RANKL up-regulation in murine osteoblasts, since an isogenic mutant strain deficient in both Lys- and Arg-gingipains exhibited a reduced capacity to induce RANKL expression, compared to its wild-type strain [45]. To investigate the putative involvement of gingipains, a Lys-X proteinase and an Arg-X proteinase mutant strain (K1A and E8, respectively) were employed to challenge the GF, and their effects were compared to those of the P. gingivalis W50 wild-type strain. Neither of the mutant strains affected OPG expression, which was comparable to that of wild-type strain. The Lys-X proteinase mutant strain did not affect RANKL regulation either, but interestingly, the Arg-X proteinase mutant strain induced an almost six-fold greater up-regulation than the wild-type strain. It is unlikely that the LPS is primarily responsible in this effect, since (a) the crucial factor appears to be a protein, and (b) the difference in LPS concentration between P. gingivalis W50 and E8 culture supernatants (Table 1) does not account for the magnitude of the observed RANKL up-regulation. Collectively, these results indicate that the Lys-gingipain is not involved in RANKL up-regulation, but the Arg-gingipains appear to have an inhibitory, or regulatory effect, since in their absence RANKL induction is more pronounced. By their enzymatic activity, Arg-gingipains have the capacity to cleave a number of host factors, including immunoglobulins, complement factors and fibronectin–integrin binding domains, but they may also have a housekeeping role in

<table>
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<tr>
<th>Strain</th>
<th>LPS concentration</th>
<th>Arg-X protease</th>
<th>Lys-X protease</th>
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<tr>
<td>W50</td>
<td>0.18</td>
<td>20.0 x 10^-3</td>
<td>7.2 x 10^-3</td>
</tr>
<tr>
<td>E8</td>
<td>0.30</td>
<td>0.3 x 10^-3</td>
<td>7.0 x 10^-3</td>
</tr>
<tr>
<td>K1A</td>
<td>0.25</td>
<td>24.4 x 10^-3</td>
<td>0.2 x 10^-3</td>
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The LPS concentration and the Arg-X and Lys-X protease activities in the culture supernatants of the three P. gingivalis strains are determined as described in Section 4.3. LPS concentration is expressed in μg per μg of bacterial protein. The proteolytic activities are expressed as units of protease activity per μg of bacterial protein.
regulating other proteins of the bacterial cell surface [6]. This explanation is further strengthened in light of recent findings demonstrating that gingipains can proteolytically inactivate other cell surface P. gingivalis proteins, which are responsible for activating TLR2- and TLR4-independent signaling pathways [47]. In this case, an Rgp/Kgp-null mutant induced more evidently these pathways in CHO cell lines, compared to its parental strain. This capacity of P. gingivalis to inactivate its own ligands via the activity of gingipains, was interpreted by the authors as a strategy by the organism to modulate immune recognition and evade host responses. Accordingly, it is tempting to speculate that P. gingivalis may as well employ its gingipains in order to regulate its virulence potential in other aspects of periodontal pathogenesis, such as the induction of RANKL, and subsequently osteoclastogenesis and bone resorption.

In conclusion, P. gingivalis secreted products regulate RANKL and OPG expression in periodontal connective tissue cells, in a manner that increases their osteoclastogenic potential. The responsible component for RANKL induction in the present experimental system appears to be a protein, which could be regulated by the Arg-gingipains. The identity of the responsible component remains to be investigated, as well as its relation to Arg-gingipains.

4. Materials and methods

4.1. Cell cultures

Human GF and PDL cells lines were established as previously described [32]. The PDL and gingival tissue biopsies used were obtained from four healthy young individuals (ages, 13–16 years), who were about to have their first premolars removed in the course of orthodontic treatment. Ethical approval was granted by the Human Studies Ethical Committee of Umeå University, Sweden (§68/03, dnr 03-029), and informed consent was given by all subjects. The cells were cultured in Minimum Essential Medium (MEM) Alpha medium (Gibco-BRL Life Technologies, UK), supplemented with 10% heat-inactivated fetal bovine serum (Bio-Whittaker, Maryland, USA), 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco-BRL Life Technologies). All cell lines were confirmed to be free of mycoplasma infections by using DAPI fluorescence staining. For the experiments, GF or PDL cells at passages 3 or 4, were seeded at concentration 20 × 10⁶ cells/cm², and allowed to attach for 24 h, maintaining a sub-confluent status. Thereafter, the cells were cultured in presence or absence of P. gingivalis bacterial culture supernatants for 6 or 24 h.

4.2. Bacteria and growth conditions

P. gingivalis W50 wild-type strain and its derivative E8 and K1A mutant strains were used in this study. E8 strain is deficient in both Arg-gingipain A and Arg-gingipain B (rgpA rgpB), whereas K1A strain is deficient in Lys- gingipain (kgp) [48]. All strains were cultured blood agar base supplemented with 5% horse blood (Oxoid, Hampshire, UK) and maintained by weekly subculture for up to 5 weeks. Liquid cultures were prepared by inoculation of bacterial colonies (3–4 days old) from blood agar plates into 10 ml brain heart infusion (BHI) broth (Oxoid) supplemented with 5 mg/l hemin (Sigma, Dorset, UK), and incubated for 24 h. Ten percent inoculum was transferred to 90 ml of the same medium and incubated for 6 days. All cultures were grown at 37°C in a Don Whitley anaerobic cabinet, MACS MG500, in an atmosphere of 80% N₂, 10% H₂, and 10% CO₂. After this culture period, bacteria were harvested by centrifugation at 10,000g for 15 min at 4°C and supernatants were collected, filter-sterilized over a 0.2 μm filter, and stored at −80°C until use.

4.3. Characterization of bacterial culture supernatants

These P. gingivalis preparations were diluted in the cell-culture medium, and their concentration is expressed as total bacterial protein (μg/ml) present in the cell cultures. Protein concentration was determined by Bio-Rad Protein assay (Bio-Rad, Hertfordshire, UK), whereas LPS concentration was determined by Limulus Amebocyte Lysate-based assay QCL-1000 (Cambrex Bio Science Walkersville, MD), according to the manufacturer's instructions, and absorbances were determined spectrophotometrically at wavelengths of 595 and 405 nm, respectively. Arg-X protease activity was measured in 0.1 M Tris–HCl (10 mM L-cysteine, 10 mM CaCl₂, pH 8.1, 30°C) with N-benzoyl-dl-arginine-p-nitroanilide (dl-BApNA) (500 mM) as the substrate. Lys-X protease activity was measured with N-ε-acetyl-L-lysine-p-nitroanilide (AcLyspNA) (250 mM) as substrate in the same reaction buffer and under the same conditions as described above. The reactions were monitored at 405 nm, and enzyme activity was expressed in units. One unit of protease activity is defined as the amount of enzyme causing an increase in the absorbance of 1.0 min⁻¹ at 30°C. The E8 and K1A strains were confirmed to have diminished Arg-X and Lys-X protease activities, respectively. The concentrations of LPS and the protease activities in the various bacterial culture supernatants are provided in Table 1.

4.4. Cytotoxicity assay

The putative cytotoxic effects of P. gingivalis on GF and PDL cell cultures were evaluated by measurement of the extracellularly released cytosolic LDH, using the CytoTox96® Non-Radioactive Cytotoxicity Assay (Promega, Southampton, UK). In brief, triplicate GF or PDL cell cultures were exposed to ascending protein concentrations of P. gingivalis supernatant for 6 and 24 h. Cell culture supernatants were thereafter collected, and the cell monolayer was lysed in equal volume of lysis buffer, provided in the kit. The collected suspensions, either cell-culture
supernatant or cell lysate, were centrifuged at 1000 rpm for 5 min to pellet down any cell debris, and thereafter 100 μl well of each was transferred into an optically clear 96-well plate. Reaction solution was added to each well and incubated for 30 min in darkness. This enzyme reaction was then stopped by the addition of 1 N HCl. The absorbance was measured at 490 nm, and background values were subtracted from all samples.

4.5. RNA extraction and cDNA synthesis

Upon termination of the experiments, the culture media were discarded, and the cell monolayers were washed twice in PBS before being lysed. The collected cell lysate was homogenized with a QIAshredder (QIAGEN, Crawley, UK), and total RNA was extracted by using the RNeasy Mini Kit (QIAGEN, Crawley, UK), according to the manufacturer’s instructions. The extracted RNA was finally eluted in 40 μl RNase free water and its concentration was determined spectrophotometrically. One microgram of total RNA was reverse transcribed into single-stranded cDNA by using M-MLV Reverse Transcriptase (RNase H Minus, Point Mutant), Oligo(dT)15 Primers, and PCR Nucleotide Mix according to the manufacturer’s protocol (all from Promega, Southampton, UK), at 40°C for 60 min, and 70°C for 15 min. The resulting cDNA was stored at −20°C until further use.

4.6. Quantitative real-time PCR

For RANKL, OPG and 18S rRNA gene expression analysis, quantitative real-time PCR was performed in an ABI Prism 7900HT Sequence Detection System and software (Applied Biosystems, Foster City, CA). 18S rRNA was used as an endogenous RNA control in the samples. The probes and the primers were synthesized by Applied Biosystems (assay IDs RANKL: Hs00243522-m1, OPG: Hs00171068-m1, and 18S rRNA: Hs99999901-s1) and the amplification reactions were performed with qPCR Master Mix (Abgene, Epsom, UK). The standard PCR conditions were 10 min at 95°C, followed 40 cycles at 95°C for 15 s, 60°C for 1 min and 72°C for 30 s. The expression levels of RANKL and OPG transcripts were calculated by using the comparative Ct method (2^(-ΔCt) formula) after normalization to 18S rRNA.

4.7. Statistical analysis

Student’s t-test for paired values was used for the statistical analyses, and data were considered significant at P < 0.05.

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References


