Factors influencing oral health in patients during cancer treatment; with emphasis on the relationship between the oral microbiome and oral mucositis

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Citation for published version (APA):

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Chapter 7

Impact of virulence factors of *Porphyromonas gingivalis* on wound healing

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submitted
Abstract

Porphyromonas gingivalis inhibited oral epithelial wound healing in vitro stronger than other oral bacteria. It is unknown why P. gingivalis is such a potent inhibitor of wound healing. Therefore, the aim of this study was to investigate the influence of major virulence factors of P. gingivalis on wound healing in an in vitro wound healing model. The influence of the capsular polysaccharide, the Arg- and Lys- gingipains, the major fimbriae and lipopolysaccharide (LPS) was investigated. A standardized scratch was made in a confluent layer of human oral epithelial cells (H0-1-N-1). The epithelial cells were then challenged with different concentrations of several P. gingivalis wild type strains and knockout mutants. Closure of the scratch was determined after 17h and compared to control conditions without the bacteria. The P. gingivalis strains ATCC 33277, W83 and W50 significantly inhibited wound healing, P. gingivalis ATCC 33277 was the most potent inhibitor of these. The presence of a capsular polysaccharide significantly lowered the inhibition of epithelial cell migration, while gingipain activity significantly increased the inhibition of cell migration. LPS and the major fimbriae did not influence epithelial cell migration. Not any of the tested P. gingivalis strains completely prevented the inhibition of cell migration. In conclusion, the capsular polysaccharide decreased and the Arg- and Lys- gingipains of P. gingivalis increased the inhibition of wound healing by P. gingivalis, while LPS and the major fimbriae had no effect. The results suggest that also other characteristics of P. gingivalis play a role in the inhibition of wound healing and further research is needed.
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Introduction

In hematopoietic stem cell transplant (HSCT) patients, the oral microflora is traditionally assumed to colonize existing ulcerative lesions rather than to cause them (1). However, recently Laheij and de Soet (2) and Stringer and Logan (3) suggested a more important role for oral microorganisms in oral mucositis. Oral microorganisms are likely able to contribute to the development of oral mucositis by a shared pathway and may prolong the existence of established ulcerations by delaying their healing.

Lately two studies described the negative influence of oral bacteria on the healing of wounds using an in vitro scratch assay (4, 5). Laheij et al (2013) studied the influence of Porphyromonas gingivalis, Prevotella nigrescens, Prevotella intermedia, Tannerella forsythia and Streptococcus mitis in different concentrations on closure of a scratch in epithelial cells from an oral epithelial cell line. Battacharya et al. (2014) studied the effect of P. gingivalis and Fusobacterium nucleatum on wound closure and on epithelial cell migration, proliferation and apoptosis in primary epithelial cells. In both studies, P. gingivalis was a potent inhibitor of re-epithelialization of the scratch.

The mechanisms by which P. gingivalis acts as a strong inhibitor of wound closure have not been studied so far. It is known that P. gingivalis has a range of virulence factors that enable it to invade into host tissues and to deregulate the immune system to promote survival inside the host (10). Important virulence factors of P. gingivalis are lipopolysaccharide (LPS), gingipain proteinases, the capsular polysaccharide and the fimbriae (11). These virulence factors are involved in the host-pathogen recognition (LPS) (12), they trigger intracellular signaling events and an immune response (LPS, gingipains) (10), they are involved in binding of P. gingivalis to host cells (gingipains, fimbriae) (10, 13) and they are involved in invasive growth (capsular polysaccharide) (14).

It was hypothesized that these virulence factors could account for the inhibiting effect that P. gingivalis had on wound healing. Therefore the aim of this study was to investigate the role of these virulence factors of P. gingivalis on wound closure by using natural variants and mutant strains in an in vitro scratch assay using oral epithelial cells.

Materials and Methods

Cell culture

Human oral buccal epithelial cell line HO-1-N-1 was provided by Japanese Collection of Research Bioresources (Osaka, Japan). HO-1-N-1 is an immortalized
epithelial cell-like cell line that originated from a squamous cell carcinoma located on the buccal mucosa. The epithelial cells were grown in tissue culture flasks with Dulbecco’s Modified Eagle’s Medium-F12 (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Hyclone, Logan, UT, USA), 2% antibiotics (penicillin, streptomycin and fungizone, Sigma, St. Louis, MO, USA) in a humidified atmosphere at 37°C with 5% CO₂. Cell culture medium was refreshed twice a week.

**Bacterial strains and cultures**

The *P. gingivalis* strains used are listed in Table 1. All *P. gingivalis* strains were grown anaerobically in Brain-Heart-Infusion (BHI; BD Difco, Le Pont de Claix, France) broth enriched with hemin (5mg/L) and menadione (1mg/L) until midlog phase. In case of mutants selection and growth, 50 µg/ml of erythromycin (Em) or 1 µg/ml tetracycline was added to the medium. Purity of the cultures was checked by Gram staining and culturing. Bacterial cultures were washed twice with Dulbecco’s Phosphate-Buffered Saline (DPBS, Invitrogen, Carlsbad, CA, USA) and resuspended in keratinocyte serum free medium (SFM) (Invitrogen, Carlsbad, CA, USA) at OD₆₉₀ 0.1 (5.10⁸ CFU/ml). Experiments were performed with either viable or heat inactivated bacteria, in which case the bacterial cultures were heated at 60°C for 60 minutes. Absence of growth on blood agar plates confirmed the killing of the bacteria.

**Lipopolysaccharide (LPS)**

The influence of LPS from *P. gingivalis* was tested by adding pure LPS from *P. gingivalis* to the epithelial cells or by blocking LPS in the cell wall of *P. gingivalis*. Ultrapure LPS from *P. gingivalis* was purchased (Invivogen, Toulouse, France) and used in the assay in a concentration of 10 µg/ml. To exclude the influence of LPS, polymyxin B (Sigma-Aldrich, St. Louis, MO, USA), an LPS inhibitor, was added to 5.10⁸ CFU/ml heat inactivated *P. gingivalis* ATCC 33277 in a concentration of 50 µg/ml.

**In vitro wound closure assay**

Cell cultures were trypsinized and seeded in 24-wells plates at 3-5.10⁵ cells/ml. When confluency was reached, a scratch with a blue sterile pipet point was made in each well. The wells were washed three times with DPBS and challenged with viable or heat inactivated bacteria or LPS from *P. gingivalis*. In control wells only SFM was added. Images were taken of the middle of the well immediately after adding the bacteria and 17 hours later with an inverted digital phase contrast microscope (Advanced Microscopy Group, Evos fl, USA).
Table 1 *P. gingivalis* strains used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Properties</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>W38 (wild type)</td>
<td>Capsular serotype 1 (K1)</td>
<td>(28)</td>
</tr>
<tr>
<td>ΔEpSC</td>
<td>Non-encapsulated (K-)</td>
<td>(21)</td>
</tr>
<tr>
<td>ATCC33277 (wild type)</td>
<td>Non-encapsulated (K-)</td>
<td></td>
</tr>
<tr>
<td>KDP 129</td>
<td>Lysine gingipain (Δkgp)</td>
<td>(29)</td>
</tr>
<tr>
<td>KDP 133</td>
<td>Arginine gingipain (Δrgp ArgpB)</td>
<td>(30)</td>
</tr>
<tr>
<td>FimA</td>
<td>Lacking major fimbriae (ΔfimA)</td>
<td>this paper, S2</td>
</tr>
<tr>
<td>W50 (wild type)</td>
<td>Capsular serotype 1 (K1)</td>
<td>(29)</td>
</tr>
<tr>
<td>K1A</td>
<td>Lysine gingipain (Δkgp)</td>
<td>(31)</td>
</tr>
<tr>
<td>E8</td>
<td>Arginine gingipain (Δrgp ArgpB)</td>
<td>(31)</td>
</tr>
<tr>
<td>HG91</td>
<td>Non-encapsulated (K-)</td>
<td>(28)</td>
</tr>
<tr>
<td>ATCC49417</td>
<td>Capsular serotype 4 (K4)</td>
<td></td>
</tr>
<tr>
<td>HG1691</td>
<td>Capsular serotype (K6)</td>
<td>Own isolate</td>
</tr>
</tbody>
</table>

The surface of the scratch was calculated with Photoshop CS4 (version 11.0.1, Adobe). Percentage of closure of the scratch was calculated as: 100 – ((surface area of the scratch at time 17h / surface area of the scratch at time 0) * 100). To account for differences in wound closure between experiments, the closure of the scratch relative to control (relative closure) was calculated by dividing the percentage of closure of the treatment with the percentage of closure of the control (SFM) treatment * 100%. Relative closure between 0-100% indicated inhibition of wound closure, with 0 indicating complete inhibition. Each treatment was performed in quintuple and each experiment was completed on three separate occasions.

**Statistics**
Differences in relative closure between the different bacterial strains and versus control were tested for significance with the non-parametric Mann-Whitney U test using SPSS version 21.0 (IBM SPSS, Chicago, IL, USA). A p-value < 0.05 was considered statistically significant.
Results

A time lapse movie of a scratch in oral epithelial cells either challenged with a high concentration of *P. gingivalis* ATCC 33277 or with medium only (control) can be found in supplemental file S1.

The heat inactivated *P. gingivalis strains* ATCC 33277, W83 and W50, significantly inhibited the migration of oral epithelial cells at all tested MOI’s compared to control in a dose responsive way (Figure 1). Heat inactivated ATCC 33277 inhibited wound closure significantly more than W83 and W50 at MOI 1000 and 100. Viable *P. gingivalis* ATCC 33277, W83 and W50, significantly inhibited the migration of oral epithelial cells at all tested MOI’s compared to control in a dose responsive way as well (Figure 1). Viable *P. gingivalis* ATCC 33277 had a more pronounced inhibiting effect on wound closure than W50, but not W83. Comparing the wound closure rates of the viable *P. gingivalis* strains to their heat inactivated variants, it showed that viable W83 and W50 inhibited cell migration more than their heat inactivated variants (except for W50 at MOI 10) however this was not the case for viable *P. gingivalis* ATCC 33277 (p>0.05).

**Figure 1** Relative closure (mean + SEM) of scratch in oral epithelial cells challenged with heat inactivated and viable *P. gingivalis* strains ATCC 33277, W83 and W50.

- a=significantly different from control (p<0.05)
- b=significantly different from MOI 1000 of the same strain (p<0.012)
- c=significantly different from MOI 100 of the same strain (p<0.001)
- * =significantly different from ATCC 33277 of the same MOI (p<0.012)
- # =significantly different from heat inactivated *P. gingivalis* of the same MOI and strain (p<0.002)
Adding pure LPS from *P. gingivalis* to the oral epithelial cells did not result in inhibition of migration of the epithelial cells (p=0.39) (Figure 2a). *P. gingivalis* cells with blocked LPS activity, by the addition of polymyxin B, did not affect relative closure of the scratch in epithelial cells differently from *P. gingivalis* alone (p=0.38) (Figure 2a).

**Figure 2** Relative closure (mean + SEM) of scratch in oral epithelial cells challenged with LPS from *P. gingivalis* and LPS inactivated *P. gingivalis* by polymyxin B (a) and heat inactivated *P. gingivalis* strains W83 and EpsC mutant (b). *p<0.01
The capsule lacking *P. gingivalis* mutant EpsC inhibited cell migration significantly more than the encapsulated wild type strain (Figure 2b). Relative closure of the scratch in oral epithelial cells challenged with *P. gingivalis* strains of different capsular serotypes is shown in Table 2. All strains inhibited epithelial cell migration (p=0.001), however not all capsular serotypes inhibit wound healing by the same rate (all MOI’s, p<0.002). The non-encapsulated strains (ATCC33277 and HG91) inhibited epithelial cell migration significantly more than the K1 and K6 capsular serotypes at all MOI’s (p<0.002), except for MOI 10 of strain HG91 vs ATCC 33277. The relative closure rates of the K4 capsular serotype was somewhat in between the other capsular serotypes. At MOI 1000 there were no statistical significant differences compared to the non-encapsulated strains, whereas at MOI 100 K4 inhibited cell migration significantly less than the HG91 strain, but not the ATCC 33277 strain. On the other hand the K4 strain inhibited wound closure significantly more than the K1 and K6 capsular serotypes at all MOI’s (p<0.001).

**Table 2** Relative closure (mean + SEM) of scratch in oral epithelial cells challenged with heat inactivated *P. gingivalis* strains with several capsular serotypes and a fimbriae lacking mutant.

<table>
<thead>
<tr>
<th>Strains</th>
<th>MOI 1000</th>
<th>MOI 100</th>
<th>MOI 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC33277 (K-)</td>
<td>0%</td>
<td>29% (+3)</td>
<td>81% (+5)</td>
</tr>
<tr>
<td>HG91 (K-)</td>
<td>0%</td>
<td>11% (+1)</td>
<td>69% (+3)</td>
</tr>
<tr>
<td>W83 (K1)</td>
<td>23% (+3)</td>
<td>70% (+5)</td>
<td>91% (+3)</td>
</tr>
<tr>
<td>ATCC49417 (K4)</td>
<td>0%</td>
<td>30% (+5)</td>
<td>72% (+6)</td>
</tr>
<tr>
<td>HG1691 (K6)</td>
<td>17% (+2)</td>
<td>48% (+4)</td>
<td></td>
</tr>
<tr>
<td>Heat inactivated FimA</td>
<td>7% (+6)</td>
<td>38% (+10)</td>
<td>85% (+6)</td>
</tr>
<tr>
<td>mutant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viable FimA mutant</td>
<td>0%</td>
<td>23% (+2)</td>
<td>75% (+5)</td>
</tr>
</tbody>
</table>

The influence of protease activity was tested using gingipain mutants constructed from both the ATCC 33277 and the W50 strain. For strain ATCC 33277 it was found that wound closure was significantly more inhibited by the wild type strain than by the both gingipains mutants (Figure 3a). There was no significant difference in relative closure between the *Kgp* and *RgpArgpB* mutants. The viable gingipain mutants inhibited wound closure stronger than the heat inactivated variants only at MOI 1000 (Figure 3a). For the W50 strain it was found that the gingipain mutants, either viable or heat inactivated, inhibited the epithelial cell migration less than the wild type strain (Figure 3b).
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**Figure 3** Relative closure (mean + SEM) of scratch in oral epithelial cells challenged with heat inactivated and viable *P. gingivalis* wild type strain ATCC 33277, ΔKgp and ΔRgpArgpB mutants (A) and heat inactivated and viable *P. gingivalis* wild type strain W50, ΔKgp and ΔRgpArgpB mutants (B).

*a* = significantly different from wild type strain of the same MOI (p<0.001)

#= significantly different from heat inactivated *P. gingivalis* of the same MOI and strain (p<0.019)

§= significantly different from the ΔKgp mutant (p<0.0)
The viable gingipain mutants significantly inhibited wound closure more than the heat inactivated variants at MOI 1000 and MOI 100 (Figure 3B). The heat inactivated RgpArgpB mutant inhibited wound closure less than the Kgp mutant, whereas the viable RgpArgpB mutant led to more inhibition of wound closure than the Kgp mutant at MOI 1000 and MOI 100 (Figure 3b).

Viable FimA mutants, lacking the major fimbriae, did not differ in inhibiting epithelial cell migration compared to the wild type strain (MOI 100: p=0.10, MOI 10: p=0.627). The heat inactivated FimA mutant did inhibit epithelial cell migration significantly less than the wild type at high concentrations (Table 2; MOI 1000: p<0.01, MOI 100: p<0.05, MOI 10: p=0.575), although the difference was very small.

Discussion

Previous research described the effect of \textit{P. gingivalis} on cell migration and wound healing finding a significant inhibiting effect of \textit{P. gingivalis}. The responsible virulent factors, however, were not determined (5, 15, 16). By comparing different naturally occurring variants and mutant strains of \textit{P. gingivalis} we studied all major virulence factors of \textit{P. gingivalis} in order to find out which of these could affect wound healing using an \textit{in vitro} wound healing model. In the \textit{in vitro} wound healing model in this research, the migration of cells is studied, which is an important step in wound healing (17, 18).

All the \textit{P. gingivalis} strains that we used in this study inhibited wound healing. The difference between viable and heat inactivated \textit{P. gingivalis} W83 and W50 suggests that there were heat resistant and heat sensitive factors of \textit{P. gingivalis} that influenced wound healing. The heat resistant virulence factors of \textit{P. gingivalis} that were expected to account for this effect were LPS, the capsular polysaccharide and the fimbriae, while the heat sensitive factors could be the gingipains (19). We found that the capsular polysaccharide and the gingipains influenced wound healing significantly, while LPS and the major fimbriae did not influence wound healing.

Until now, only the effect of the capsule on the immune system and on human cells was described. Both stimulating and inhibiting effects were reported (20-22). In the present experiments, the non-encapsulated bacteria inhibited wound closure stronger than the encapsulated bacteria. Not only the presence, but also the thickness of the capsule seemed to be important. The K4 serotype has a relatively thin capsule, while the K1 and the K6 serotypes have significantly thicker capsules (23). The K4 \textit{P. gingivalis} strain had a relative closure that was in between the K- and the K1-K6 serotypes. This can be
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explained by assuming that a capsule may hide cell-wall bound factors that might influence cell migration such as protease activity of the gingipains or other, yet unknown, antigens on the cell wall of P. gingivalis.

Gingipains are important for the adherence of P. gingivalis to epithelial cells (13). The absence of specific protease activity of the gingipain mutants used in this study were confirmed using the method that was described by Kaman et al (24) (data not shown). The protease activity of the gingipains of P. gingivalis was an important determinant of epithelial cell migration. Knocking out the gingipain activity led to improved wound closure, suggesting that gingipain activity inhibited cell migration.

The viable gingipain mutants inhibited epithelial cell migration less strong than the heat inactivated gingipain mutants. The gingipains account for 85% of the protease activity of P. gingivalis (25). Furthermore, in the RgpArgpB mutant the kgp gene is still functional and in the Kgp mutant the RgpArgpB gene is still in function. Thus, these viable gingipain mutants still possessed some protease activity, while the heat treatment inactivated almost all protease activity (19). Furthermore, gingipains have a function in the modification of several cell wall components (26). Therefore, P. gingivalis that lacks arginine or lysine gingipain activity may have a slightly different cell wall composition compared to the wild type, which may lead to differences in attachment to or recognition by the epithelial cells. This effect would have been more pronounced in the experiments with the viable bacteria, which may contribute to differences in relative closure between experiments with heat inactivated and viable bacteria found in this study.

LPS did not influence epithelial cell migration. The present results are in agreement to those of Kraus et al. (27), who found no significant differences between LPS from P. gingivalis and control, in wound healing of primary epithelial cells.

The fimbriae are important for the adherence of P. gingivalis to epithelial cells (10) and the inhibition of the fimbriae on wound healing was described by Nakagawa et al. (16). Therefore our hypothesis was that the absence of major fimbriae would lead to less inhibition of wound healing. In our experiments however, we proofed that the fimA gene was not involved in wound healing as we found no difference in relative closure between the viable wild type strain and the fimA mutant. When epithelial cells were challenged with heat inactivated bacteria, we did find significantly less inhibition of wound healing of the fimA mutant strain compared to the wild type, but the difference was small. This difference may not be biologically relevant. We could not confirm the importance of fimbriae in the inhibition of wound healing as
described by Nakagawa et al (16). This might be explained by the difference in the cell lines that were used. Nakagawa et al. used HeLa cells of cervical epithelial origin. HeLa cells may react differently on a challenge with *P. gingivalis* than our oral epithelial cell line. Moreover, the amount of wound closure Nakagawa et al. reported was generally lower.

Since there was even in the presence of a capsule and the absence of either the Arg- or Lys- gingipains, an inhibition in wound healing we hypothesize that there must be other, yet unknown, antigens on the cell wall of *P. gingivalis* that are responsible for the inhibiting effect of *P. gingivalis*. In conclusion, the *P. gingivalis* ATCC 33277, W83 and W50 strains strongly inhibited wound healing. Part of that effect was accounted for by the absence of a capsular polysaccharide and the presence of Arg- and Lys- gingipain activity. LPS and the major fimbriae did not influence wound healing. Since not all inhibition could be explained by the presence of capsular polysaccharide or the Arg- and Lys- gingipains, other yet unknown characteristics of *P. gingivalis* must have contributed to this effect and further research is necessary.

**Acknowledgements**

All authors declare no conflict of interest. 
dr. F. Bikker is gratefully acknowledged for providing the FRET assay. K. Hoebe is gratefully acknowledged for providing the TEM images.
References


Supplemental file S1
http://www.onderwijs.acta.nl/ictowerkgroep/support/S1timelapsemovie.pptx
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**Supplemental file S2**

The *fimA* mutant strain was constructed by a PCR ligation mutagenesis method. In details, genomic DNA from *P. gingivalis* ATCC33277 was isolated from an overnight culture with GeneJet genomic DNA purification kit (Thermo Scientific) and used as a template for PCR. The flanking regions of the *fimA* gene were amplified from the genomic DNA with two PCR primer pairs:

- fimAuf/fimAur (fimAuf 5’-AGGCTATGCTTCGCCGGAAGGT-3’; fimAur 5’-GGCGCGCCCAAGAGCAGCAAGCAAGTCCCAACAAGA-3’) and
- fimAdf/fimAdr (fimAdf 5’-ACTAGTTGCTACTTGGTAATCGACCCGTCAAA-3’; fimAdr 5’-GATGCAGAGGAGTAGATCAAGTGT-3’).

The erythromycin resistance gene (Em gene) was amplified from plasmid pEP4351 with primer pairs Emf/Emr (; Emf 5’-ACTAGTTGCTACTTGGTAATCGACCCGTCAAA-3’; Emr 5’ GGCGCGCCCAAGAGCAGCAAGCAAGTCCCAACAAGA-3’).

The upstream fragment was digested with Ascl and the downstream fragment was digested with SpeI. The Em fragment was double digested with Ascl and SpeI. These three digested fragments were ligated and used as the template for the amplification of a mutant construct using the primers fimAuf/fimAdr. The resulting PCR product was purified and transformed directly into *P. gingivalis* cells by electroporation. The *fimA* mutant strain was obtained via double crossover. As a result, the major part of the chromosomal *fimA* gene was replaced by the Em gene. The insertion of the Em gene was verified by erythromycin resistance and by PCR. Electron micrograph (TEM) shows the cell surface of *fimA* mutant strain that lacks the long fimbrial structure.