Human β-defensin 2 and protease activated receptor-2 expression in patients with chronic periodontitis

Alexandre Lustosa Pereira a,*, Marinella Holzhausen b, Gilson César Nobre Franco a, Sheila Cavalca Cortelli a, José Roberto Cortelli a

a Department of Periodontology, University of Taubaté, Taubaté, SP, Brazil
b Department of Periodontics, Department of Stomatology, School of Dentistry, University of São Paulo, São Paulo, SP, Brazil

A R T I C L E   I N F O

Article history:
Accepted 26 April 2012

Keywords:
Chronic periodontitis
Protease activated receptor-2
Human beta defensin 2
Periodontal treatment

A B S T R A C T

Objective: Some previous studies have shown that gingipains, trypsin-like proteases produced by Porphyromonas gingivalis, up-regulate human β defensin-2 (HBD-2) mRNA expression through protease-activated receptor-2 (PAR2) in gingival epithelial cells. This study aimed at investigating salivary HBD-2 levels and crevicular PAR2 mRNA expression in human chronic periodontitis and evaluating whether periodontal treatment affected this process.

Methods: Salivary and gingival crevicular fluid (GCF) samples were collected from periodontally healthy (control) and chronic periodontitis patients at baseline and 50 days after non-surgical periodontal treatment. Salivary HBD-2, and GCF TNF-α levels were analysed by ELISA, and PAR2 mRNA at the GCF was evaluated by RT-PCR.

Results: P. gingivalis was significantly (p < 0.05) more prevalent in patients with chronic periodontitis when compared to controls. This prevalence decreased after periodontal therapy (p < 0.0001). The control group showed statistically significant lower levels of HBD-2, TNF-α, and PAR2 expression when compared to the chronic periodontitis group. In addition, periodontal treatment significantly reduced PAR2 expression and HBD-2 levels in chronic periodontitis patients (p < 0.001).

Conclusions: Our results suggest that salivary HBD-2 levels and PAR2 mRNA expression from GCF are higher in subjects with chronic periodontitis than in healthy subjects, and that periodontal treatment decreases both HBD-2 levels and PAR2 expression.

© 2012 Elsevier Ltd. Open access under the Elsevier OA license.

1. Introduction

Human β-defensins (HBDs) are small cationic peptides produced throughout the body, mainly by epithelial cells, that play an important role in the oral cavity as a first-line defence against gram-negative and gram-positive bacteria, as they are able to create pores into the bacterial membranes, killing the bacteria. Epithelial cells in the oral cavity constitutively express HBDs: HBD-1, HBD-2, and HBD-3.1,2 However, in the presence of inflammation, a different expression of these peptides might occur.2–5 Dommisch et al.3 showed that in healthy gingival tissues there is a similar expression among HBD-1 and -2 mRNA. In contrast, the expression of HBD-2 is statistically higher than human β defensin-1 in both gingivitis and chronic periodontitis subjects.
A recent study by Vardar-Sengul et al. showed that the expression of HBD-1 and -2 mRNA was significantly higher in chronic periodontitis subjects than in the healthy control group. In addition, in a study by Kuula et al., HBD-2 expression was found to be lower in periodontally healthy tissues than in inflamed periodontal and peri-implant tissues. Taken together, these studies suggest a potentially important role for defensins in the host response to infection by periodontal pathogens.

The modulation of the β-defensins expression in the oral cavity can be orchestrated by receptors present in the cell membrane that recognize certain molecular patterns associated with periodontal pathogens, including Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, and Fusobacterium nucleatum. Previous in vitro studies have shown that gingipains, trypsin-like proteases produced by P. gingivalis, upregulate HBD-2 mRNA expression through protease-activated receptor-2 (PAR2) in gingival epithelial cells. PAR2 belongs to the family of G-protein-coupled, seven-transmembrane-domain receptors. Its activation occurs through the proteolytic cleavage of the N-terminal domain by serine proteinases such as trypsin, mast cell tryptase, neutrophil proteinase 3, tissue factor/factor VIIa/factor Xa, membrane-tethered serine proteinase-1, and gingipains. A recent study by our group compared chronic periodontitis patients to healthy controls and showed that PAR2 is up-regulated in this first group. We also showed that the presence of P. gingivalis in the periodontal pocket is associated with this upregulation of PAR2 gene expression and that a higher pro-inflammatory profile is related to advanced periodontal destruction. In the present study, we hypothesized that HBD-2 levels as well as the expression of PAR2 are elevated in the saliva of chronic periodontitis subjects. As to assess this hypothesis, the salivary HBD-2 levels and the PAR2 mRNA expression from GCF were investigated in chronic periodontitis and in healthy subjects. In addition we also evaluated whether periodontal treatment may affect these levels.

2. Materials and methods

2.1. Patients

All subjects were between 20 and 69 years and in good overall health. Patients who reported history of tobacco usage within six months of screening; use of orthodontic appliances; need for premedication with antibiotics for dental treatment; usage of antibiotics, phenytoin, calcium antagonists, cyclosporine, or anti-inflammatory drugs within one month of initial appointment; history of any disease known to compromise immune functions; pregnancy or lactation; immunosuppressive chemotherapy, and/or periodontal treatment within the last 6 months, were not included in the present study.

The study protocol was approved by the Institutional Committee on Research of the University of Taubate (protocol #385/08) in accordance with the Helsinki Declaration of 1975, as revised in 2000. All patients were instructed in the nature and objectives of the study and signed a consent form agreeing to their participation.

2.2. Clinical examination

Subjects were clinically evaluated with regards to the probing pocket depths, clinical attachment loss and bleeding upon probing recorded at six sites per tooth (mesiobuccal, buccal, distobuccal, mesiolingual, lingual, distolingual) using a manual periodontal probe (PCPUNC 15 – Hu-Friedy, Chicago, IL, USA). Patients were then divided in two groups: control (i), formed by those showing healthy sites with probing depths of ≤3 mm, no attachment loss, no bleeding on probing, or no signs of inflammation (10 subjects); and chronic periodontitis (ii), formed by patients with at least four teeth with one or more sites with probing depth ≥4 mm and clinical attachment loss ≥3 mm, and bleeding on probing. For each patient in the chronic periodontitis group the periodontal site showing the deepest probing depth in each oral quadrant was selected for the collection of microbial and GCF samples.

2.3. Periodontal therapy

After the clinical evaluation, bacterial and saliva samples were taken. Each subject received oral hygiene instructions and a standard kit for mechanical supragingival plaque control. The kit contained fluoride dentifrice, a regular toothbrush, interdental toothbrushes, and dental floss. Subjects in the healthy group were instructed about personal daily oral hygiene care. Periodontitis subjects underwent scaling and root planning under local anaesthesia, in a total of four clinical visits.

Clinical data, microbial, crevicular fluid and salivary samples were taken from the same sites at baseline and 50 days after initial therapy.

2.4. Microbial sampling and P. gingivalis analysis

The periodontal sites selected were isolated and the supragingival plaque was carefully removed. One fine paper point (number 30 – Tanari – Tanariman Industrial Ltd., Manacapuru, Brazil) was inserted into the gingival sulcus/periodontal pocket and left in place for 10 s. Samples collected were stored in 1 mL of reduced Ringer’s solution (0.9 g sodium chloride, 0.042 g potassium chloride, 0.025 g calcium chloride, 100 mL distilled water) at ~80°C. Bacterial suspensions were thawed, centrifuged at 12,000 × g for 1 min. The presence of P. gingivalis was assessed by polymerase chain reaction (PCR) using specific primers: sense 5’AGGCAGCTTTGACATACTGCGG3’, and anti-sense: 5’-ACTGTAGCCTAACTACGGAT-3’ (product size: 404 bp) under standard conditions. DNA was extracted using PureLink® Genomic DNA Kit (Invitrogen, Carlsbad, CA, USA). PCR was performed in a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany) as follows: one cycle 94°C for 5 min, 35 cycles 94°C for 30 s, 57°C for 30 s, 72°C for 1 min, and a final extension of 72°C for 5 min.

After electrophoresis in 1.5% agarose gel, DNA fragments were stained with SYBR SafeTM (Invitrogen®, Carlsbad, CA, USA) and visualized by UV illumination. PCR amplifications were compared with both positive and negative controls. Molecular weight marker (Ladder 100, Invitrogen) was added in each set.
2.5. Gingival crevicular fluid (GCF) sampling

GCF samples were obtained from the same periodontal sites selected for microbial sampling. One strip of perio-paper (PerioCol Collection Strip, Oraflow, Plainview, NY, USA) was inserted into the gingival crevice/periodontal pocket and removed after 30 s. The volume of the fluid was determined using a moisture metre (Periotron 6000, IDE Interstate, Amityville, NY, USA). After that, all perio-paper strips were placed in tubes containing 500 μl of sterile 0.01 M sodium phosphate buffer, pH 7.4, and vortex mixed for 30 s. Samples were then centrifuged for 10 min at 6000 × g, and supernatant was collected and stored at −80°C. The cell pellet was stored in RNA stabilization solution (Trizol, Invitrogen, Carlsbad, CA, USA) at −80°C.

2.6. TNF-α levels in the GCF

Tumour necrosis factor-α levels were determined by using commercially available enzyme-linked immunosorbant assays (R&D Systems, Minneapolis, MN, USA). The concentration of the inflammatory mediators was calculated using the Softmax data analysis program (Molecular Devices, Menlo Park, CA, USA).

2.7. PAR2 gene expression by reverse-transcription PCR (RT-PCR)

Total RNA was isolated from the cell pellet of the GCF by the one-step method, using phenol and chloroform/isoamylalcohol. RNA was reverse-transcribed into cDNA by using the ready-to-go RT-PCR beads kit (Amersham Biosciences, Buckinghamshire, UK). Briefly, 2 μg of total RNA was used and the reaction included the random primer p(dN)6. After reverse transcription according to manufacturers’ instructions, PCR amplification was performed with the addition of specific primers. For PAR2, upstream: 5′-TGGGTTTGGCCAACTACGCCG-3′, and downstream: 5′-GGGAGATGCCAATGGAATG-3′. For GAPDH, upstream: 5′-GGTATCGTGGAAGGACTCATGAC-3′, and downstream, ATGCCAGTGAGCTTCCCGTTCAGC-3′. PCR products were loaded in 1.5% agarose gel and, 26 μL of PAR2 or GAPDH PCR products were loaded for each sample. The sizes of the amplified fragments were 324 bp and 189 bp for PAR2 and GAPDH, respectively. Amplified samples were visualized under UV light after being stained with SYBR SafeTM (Invitrogen®, Carlsbad, CA, USA). Results are expressed as PAR2 to GAPDH ratios.

2.8. Saliva sampling

Saliva samples were collected from all individuals. Samples taken from subjects in the control group were collected at baseline, whereas individuals in the chronic periodontitis group were subjected to two saliva sample collections, one at baseline, previous to periodontal therapy, and the other fifty days after comprehensive periodontal therapy. During the procedure, subjects were instructed to rinse their mouth with water and chew a piece of sterilized rubber tourniquet to stimulate saliva, which was collected to yield a total 1.0 mL. Samples were centrifuged for 10 min at 15,000 × g at 4°C, and the supernatants were immediately stored at −80°C.

2.9. ELISA for human β-defensin 2 (HBD-2)

The quantification of HBD-2 in saliva was done by an Enzyme Linked Imunosorbent Assay – ELISA (Peprotech, Rocky Hill, NJ, USA) according to manufacturer’s instructions. The process was carried as follows: 100 μL (0.25 μg/mL) of specific antibody (anti-HBD-2) was added to the 96-well polystyrene ELISA plates and incubated overnight (4°C); after being washed four times with PBST (PBS with 0.05% Tween-20), 300 μL of a blocking solution (1% BSA in PBST) was added to the wells and incubated for 1 h at room temperature. Plates were then washed and 100 μL of the samples or standards were added into the respective wells in duplicate and these plates were incubated for 2 h. After washing, 100 μL of detection antibody (0.5 μg/mL) was applied to the wells and plates were incubated for 2 h. After this period, plates were washed and 100 μL of streptavidin-conjugated horseradish peroxidase (1:2000 in PBST) was added to the respective wells and incubated for 30 min. Colorimetric reactions were developed using o-phenylenediamine in the presence of 0.02% H2O2. Reaction was stopped using H2SO4 (2N) and measured by an ELISA reader (OD 490 nm).

2.10. Statistical analysis

One-way analysis of variance was used to compare means among groups. In case of significant differences among groups, post hoc two-group comparisons were assessed with a Tukey-Kramer test. The prevalence of P. gingivalis among groups was analysed using the chi-square test. A p value < 0.05 was considered statistically significant. Data are expressed as mean ± SE.

<table>
<thead>
<tr>
<th>Table 1 – Clinical parameters.</th>
<th>PD (mm)</th>
<th>±SD</th>
<th>CAL (mm)</th>
<th>±SD</th>
<th>PI (%)</th>
<th>±SD</th>
<th>GI (%)</th>
<th>±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control N = 10</td>
<td>1.02</td>
<td>0.21</td>
<td>0.95</td>
<td>0.4</td>
<td>38.11</td>
<td>11.9</td>
<td>18.46</td>
<td>9.32</td>
</tr>
<tr>
<td>Chronic periodontitis (CP) N = 10</td>
<td>3.22</td>
<td>0.55</td>
<td>2.32</td>
<td>1</td>
<td>78.81</td>
<td>19.31</td>
<td>72.53</td>
<td>18.68</td>
</tr>
<tr>
<td>CP post-treatment (CPT) N = 10</td>
<td>2.4b</td>
<td>0.36</td>
<td>2.03b</td>
<td>1.26</td>
<td>22.3b</td>
<td>13.13</td>
<td>23.5b</td>
<td>11.19</td>
</tr>
</tbody>
</table>

N: number of subjects; SD: standard deviation; PD: mean probing depth; CAL: mean clinical attachment level; PI: plaque index; GI: gingival index. Number of sites > 4 mm on CP-group: 493 of 1326, i.e., about 37% of overall.

Statistically different compared to control group, p < 0.05.

Statistically different compared to CP group, p < 0.05.
3. Results

3.1. Clinical findings

Mean pocket depth (PD) and mean clinical attachment loss (CAL) were significantly higher \((p < 0.05)\) in subjects in the chronic periodontitis group than in those in control. Clinical parameters were significantly \((p < 0.05)\) improved by conventional periodontal treatment (Table 1).

3.2. Laboratory findings

Patients with chronic periodontitis showed a significant increase \((p < 0.001)\) in the mean PAR2 mRNA expression relative to the GAPDH RT-PCR signal. Moreover, conventional periodontal treatment significantly \((p < 0.05)\) decreased PAR2 mRNA expression (Fig. 1A).

Although being significantly \((p < 0.05)\) more prevalent in patients with chronic periodontitis than in those in the control group, the levels of \(P.\) gingivalis decreased after periodontal therapy \((p < 0.0001)\) (Fig. 1B).

Levels of TNF-\(\alpha\), that were also higher \((p < 0.01)\) in chronic periodontitis patients also decreased after periodontal therapy \((p < 0.001)\) (Fig. 2A).

The same was observed for the mean HBD-2 salivary levels that were higher in chronic periodontitis subjects than in controls (Fig. 2B) and that significantly decreased after therapy \((p < 0.05)\).

4. Discussion

While epithelial tissues from gut, trachea and skin only express human beta defensin-2 in the presence of infection or inflammation, the oral epithelium expresses the peptide in normal healthy gingival tissue.\(^{12}\) HBD-2 expression in normal oral epithelium is due to the constant stimulation of the innate immune response by commensal, non-pathogenic bacteria.\(^{13}\) In the normal gingival tissue, peptides are detected in the upper spinous, granular, and cornified layers, while mRNA is more strongly expressed in the spinous layer of the tissue. In the presence of pathogenic bacteria, upregulation of HBD-2
expression occurs at the gingival margin, adjacent to the biofilm in the inflamed epithelium.12

The nature of the epithelial cell receptors which are able to detect microorganisms and induce the production of the antimicrobial peptides is still not well known. Although we already understand that toll-like receptors 2 and 4 can recognize gram positive and gram negative bacteria resulting in the activation of transcriptional factors that mediate several innate and inflammatory responses,14,15 there has not been any convincing evidence of their involvement in the regulation of HBD-2 in oral epithelial cells.16

Protease-activated receptor (PAR) is another family of membrane receptors17 that probably play a role in the inflammatory and host defence response to pathogenic bacteria, including the modulation of human b defensins.18 PAR may be activated in the oral cavity through its proteolytic cleavage by P. ginvialis bacterial proteases.19 Our results demonstrated that, when compared to periodontally healthy individuals, chronic periodontitis patients show statistically significant higher levels of HBD-2 and an upregulation of PAR2. Besides, we also observed that periodontal treatment significantly reduced PAR2 expression and human b defensin-2 levels in chronic periodontitis patients (p < 0.001).

We have previously demonstrated that in subjects with chronic periodontitis a higher expression of PAR2 in the gingival crevicular fluid was associated with higher levels of pro-inflammatory mediators, total proteolytic activity, P. ginvialis prevalence and neutrophil-protease 3 mRNA expression.10 Another study by our group showed that the presence of P. ginvialis in the periodontal pocket of chronic periodontitis patients is associated with higher proteolytic activity, and a marked increased expression of PAR2.11 These evidences suggest that PAR2 plays an important role in the pathogenesis of periodontal disease in response to proteases secreted by P. ginvialis.

Chung et al.7 demonstrated that bacterial proteases such as gingipains from P. ginvialis induced expression of human b defensins in human gingival epithelial cells by activating PAR2. Furthermore, Barrera et al.20 showed that proteolytic antibodies present in the human milk may activate PAR2, which in turn induces HBD-2 expression. The exact mechanism(s) by which PAR2 is associated with an increase in HBD-2 levels remains to be established in further studies. A recent study by Lee et al.21 showed that PAR2 activation by proteases secreted by Propionibacterium acnes leads to both TNF-α and HBD-2 mRNA expression in acne lesions. Accordingly, Shin and Choi22 showed that Treponema denticola suppresses the expression of HBD-2 in gingival epithelial cells by inhibiting TNF-α production. Interestingly, in the present study, increased prevalence of P. ginvialis and levels of TNF-α were associated with higher salivary levels of HBD-2 in chronic periodontitis. In addition, periodontal treatment resulted in lower levels of both TNF-alpha and human β-defensin associated with a decreased prevalence of P. ginvialis. These evidences suggest the hypothesis that PAR2 activation by gingipains mediates the increased production of TNF-α, therefore leading to increased human β expression in chronic periodontitis.

Several studies have demonstrated that elevated levels of human β defensins are present in saliva and periodontal tissues of patients with gingivitis, periodontitis, and peri-implantitis.1-5 This is, as far as we know, the first study to show that after periodontal treatment the salivary levels of HBD-2 are decreased and associated with a decreased expression of PAR2. The exact role of PAR2 on human periodontal inflammation is still not clearly defined; however, it seems likely that it might play an important role in innate immune defence during periodontal disease by leading to the production of anti-bacterial peptides and pro-inflammatory mediators.

In conclusion, Our results suggest that salivary HBD-2 levels and PAR2 mRNA expression from GCF are higher in subjects with chronic periodontitis than in healthy subjects, and that periodontal treatment decreases both HBD-2 levels and PAR2 expression. Thus, anti-bacterial peptides production might be an important role played by PAR2 in innate immune defence during periodontal disease.

Funding

This study was supported by State University of São Paulo Research Foundation, São Paulo, Brazil (FAPESP) Research Grant 07/50665-8 to MH.

Conflict of interest

The authors have no conflict of interest or competing financial interest with regards to this manuscript.

Ethical approval

Ethical approval given from Institutional Committee on Research Involving Human Subjects of the University of Taubate # 386/08 on August 28, 2008.

Acknowledgement

The authors thank Juliana Guimarães dos Santos for technical assistance.

References

4. Vardar-Sengul S, Demirci T, Sen BH, Erkizan V, Kurulgan E, Baylas H. Human beta defensin-1 and -2 expression in the


