The expression of hBDs in the gingival tissue and keratinocytes from healthy subjects and periodontitis patients

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A B S T R A C T

Objective: Although the secretion of antimicrobial peptides in gingival tissue and isolated cells has been reported, the induction of human β-defensins (hBDs) in epithelial cells from the periodontitis patients was not stated before. This study aimed to compare the secretion of hBDs in gingival epithelial cells from periodontitis patients and healthy controls.

Design: Firstly, gingival biopsies were obtained from chronic periodontitis patients and healthy controls and the hBDs expression level in gingival tissues was quantified. Then the epithelial cells from periodontitis patients and healthy controls were isolated and challenged with different concentrations of tumour necrosis factor-alpha (TNFα). The hBDs expression level was also quantified after induction. At last, to identify the molecular pathways involved in hBDs induction, the isolated cells were incubated with NF-κB or MAPK inhibitor before TNFα induction.

Results: Higher hBDs expression was found in gingival tissues from healthy controls. The in vitro experiments demonstrated that the hBD-2 expression in gingival epithelial cells from periodontitis patients can be induced by TNFα at lower dose, while the optimum expression level was much lower. The basal hBD-3 mRNA expression was much higher in cells from periodontitis patients. The molecular pathways involved in the responses to the inflammatory cytokine in patients and healthy controls were the same.

Conclusions: The epithelial cells from periodontitis patients are more prone to recognize and respond to TNFα to produce hBD-2. The basal expression of hBD-3 in keratinocytes from periodontitis patients suggested that hBD-3 may play an important role in the immunological reaction against periodontitis.

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1. Introduction

Human gingiva is covered by the stratified squamous epithelium and the epithelium is constantly challenged by local stimuli. Except its physical barrier function, the gingival epithelium also provides protection against microbial invasion by secreting an array of antimicrobial peptides, such as human β-defensins (hBDs).1–3 hBDs are a group of small, cationic, cysteine-rich peptides with potent antimicrobial activity against both gram-positive and gram-negative bacteria, fungi, and viruses.4 Therefore the gingival epithelium plays a crucial role in the local challenge and also in the inflammatory response in periodontal diseases.

Periodontitis is one of the most common chronic infections that lead to the destruction of tooth-supporting tissues.5 The association between oral hBDs levels in gingival biopsies and periodontal diseased tissue has been published in the past few years.6–11 Studies have suggested that the changes of hBD levels could be associated with the severity of periodontal disease due to the presence of local inflammation.6–8 Some studies have demonstrated that hBD-2 mRNA expression were higher in patients with chronic periodontitis when compared with the control group,10 while others found lower hBDs expression in chronic periodontitis patients.6–8,11,12

The hBDs expression in isolated epithelial cells responsible for the stimulation has also been widely investigated.1,13–16 Studies in gingival keratinocytes models have shown that hBD-1 is constitutively expressed; while hBD-2 and hBD-3 expression can be induced by different inflammatory cytokines and microorganisms. The host response is an important determinant in periodontitis development. Therefore, we hypothesized that the response of the isolated gingival epithelial cells from periodontitis patients was in a different manner compared with those cells from healthy controls in vitro. In the present study the mRNA expression levels of hBD-1,-2,-3 in the gingival tissue and in keratinocytes from healthy subjects and periodontitis patients were evaluated. Several authors have demonstrated high concentrations of TNF-α in gingival crevicular fluid at sites with active periodontal destruction.17 So in the present study, TNF-α was chosen as a stimulus when the hBDs expression in isolated gingival epithelial cells was measured.

2. Materials and methods

2.1 Patients and biopsies

Twenty subjects were selected from the population referred to the Department of Stomatology Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. All tissue samples were obtained in compliance with a protocol approved by the Medical Institutional Review Board in Union Hospital for the Use of Human Subjects in Research. Individuals recruited included 10 healthy controls without periodontal disease and 10 patients with chronic periodontitis. The inclusion criteria for healthy controls comprised those without clinical manifestations of periodontal disease and with at least 20 natural teeth present. No record of periodontal treatment and no history of systemic disease were found.8 The patient age ranges from 20 to 60 years. Inclusion criteria of chronic periodontitis comprised the patients with at least 20 natural teeth; a minimum of six periodontal pockets >4 mm that bled on probing; and clinical attachment loss of >1 mm. The periapical radiographs revealed crestal bone loss, which was detected at a distance > 3 mm between the alveolar crest and the cemento–enamel junction around the affected teeth. The patient age ranges also from 20 to 60 years. Periodontally diseased tissues were obtained from the subjects who were undergoing periodontal surgery. Samples from the healthy controls were obtained during tooth-extraction operations.

2.2 Total RNA isolation and real-time-PCR

Totally ten gingival tissues (five samples from patients and five from controls) were flash frozen in liquid nitrogen and stored at −80 °C before use. Total RNA was isolated using RNA extraction Minikit according to the manufacturer’s protocol. The frozen gingival tissue was ground to a fine powder in a mortar that was pre-cooled with liquid nitrogen to avoid RNA degradation. All samples were treated with DNase I to remove genomic DNA contamination. RNA concentration and purity were measured photometrically using an Eppendorf Biophotometer. Total RNA was reverse transcribed into cDNA by using the cDNA synthesis Kit.

Real-time PCR was performed to determine the quantification of hBDs gene expressions using specific primers as shown before.14 The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was amplified in parallel with the interest gene. The gene expression was verified by melting curve analysis. The comparative relative copy numbers were calculated by ΔΔCT method14 conducted with a fluorescence ABI PRISM 7000 sequence detector. The cDNA amplification was in a 10ul reaction mixture containing 50 ng cDNA solution, 5ul of SYBR premix EX Taq (TAKARA, Japan), 4 pmol each of the forward and reverse primers, and 2 pmol of Rox Reference Dye II (TAKARA, Japan) in triplicate under the following conditions: an initial denaturation at 94 °C for 1 min followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 33 s.15

2.3 Gingival epithelial cells isolation and induction

Gingival epithelial cells were obtained from gingival biopsies (totally 5 samples from periodontitis group and 5 from healthy groups were included) in both groups. In brief, the ten biopsies were cut into pieces and incubated overnight at 4 °C in 2.4 U dispase II to separate the epithelium from the connective tissue. On the following day, the separated epithelium was treated with EDTA (0.02%)-trypsin (0.05%) solution for 10 min. The obtained single cell suspension was cultured in a defined serum-free medium (SFM). Cells after second passage were trypsinized, collected for the following experiments.14

To assess the induction effect of TNFα on hBDs, gingival keratinocytes from both groups were grown in six-well plates to about 85% confluence and then exposed for 24 h to TNF–α at the concentrations of 10, 50, 100, 150, 200 ng/ml. In order to study the molecular pathways involved in the present study,
NF-κB inhibitor BAY-11-7082 was used at a concentration of 10 μmol/L. BAY-11-7082 is a specific and potent inhibitor of NF-κB, which inhibits the phosphorylation and degradation of IkBa, and prevents the translocation of p65 and p50. To investigate the impact of the MAPK pathway on hBDs expression, p38 MAP kinase inhibitor SB 203580 was applied at a concentration of 10 μmol/L. 2 h before the induction of TNFα (50 ng/ml). After 24 h, the inductions were terminated, and total RNA was isolated.

2.4. RNA isolation and real-time quantitative PCR

Total RNA was extracted from stimulated keratinocytes using RNA extraction kit according to the manufacturer’s suggestion. Reverse transcription and real-time PCR were conducted as described before in gingival tissue samples. All the assays were performed in triplicate and repeated at least two times.

2.5. Statistical analysis

A statistical software (SPSS 17.0) was used to perform a t-test for the comparison of total mRNA expression in gingival tissue from periodontitis patients and healthy controls. For TNFα regulation studies, the comparison of the inducible potency was analyzed using one-way analysis of variance (ANOVA). For all analyses a P-value ≤ 0.05 was accepted as statistically significant.

3. Results

3.1. The mRNA expression of hBDs in gingival biopsies from periodontitis patients and healthy subjects

Gingival samples were collected from 5 healthy subjects and 5 patients with chronic periodontitis and real-time PCR was utilized to quantify the expression level of hBDs. Results showed that significantly lower hBD-1, -2 and -3 mRNA expression levels were observed in the chronic periodontitis lesions, when compared with healthy subjects (Fig. 1).

Fig. 1 – The mRNA expression of hBDs in gingival tissue biopsies from periodontitis patients and healthy subjects. Gingival tissue biopsies were obtained from healthy subject (n = 5) and periodontitis patients (n = 5). Total RNA was extracted and the mRNA expressions of hBDs were analyzed by quantitative PCR. * represent P ≤ 0.05, ** represent P ≤ 0.01

3.2. Induced hBDs expression in gingival epithelial cells by TNFα at different concentrations

In the present study, we hypothesized that the response of the isolated gingival epithelial cells from periodontitis patients was in a different manner compared with those from healthy subjects. The isolated epithelial cells from both groups were induced by TNFα at different concentrations and real-time PCR analysis was performed to quantify the expression level of hBDs.

Results showed that the induction profiles of hBD-1, -2 and -3 in the isolated epithelial cells from periodontitis patients were different from those of the healthy controls. The basal hBD-1 expression in these two groups was similar. The hBD-1 expression was up-regulated by TNFα at the concentration of 100, 150 and 200 ng/ml in the healthy group (Fig. 2). By contrast, the hBD-1 expression was not up-regulated or down-regulated by TNFα in the periodontitis group (Fig. 2).

Fig. 2 – Induced hBDs mRNA expression in isolated gingival epithelial cells by TNFα at different concentrations. The primary epithelial cells were obtained from periodontitis patients (n = 5) and healthy subjects (n = 5). The cells were challenged with different concentrations of TNFα (10 ng/ml, 50 ng/ml, 100 ng/ml, 150 ng/ml, 200 ng/ml). Induced mRNA expression levels of hBDs in two groups were measured by real-time quantitative PCR and compared. The asterisks in each column refer to the intragroup differences compared with their own controls. The differences between cells from periodontitis patients and healthy subjects were also shown in the graph. * represent P ≤ 0.05, ** represent P ≤ 0.01
Fig. 3 – NF-κB or MAPK pathway played an important role in the induction of hBDs. Characterization of hBDs expression (hBD-1 from healthy subjects (A), hBD-1 from patients (B), hBD-2 from healthy subjects (C), hBD-2 from patients (D), hBD-3 from control subjects (E), hBD-3 from patients (F)) in primary epithelial cells induced by TNFα with or without MAPK or NF-κB signalling pathway inhibitors was shown. Primary gingival keratinocytes were pre-incubated with SB 203580 (10 μM) or BAY-11-7082 (10 μM) for 2 h and subsequently stimulated with TNFα (50 ng/ml) for 24 h. After incubation total RNA was extracted and subjected to quantitative PCR as described in materials and methods. The graph represents the average ± standard deviation. * represent \( P \leq 0.05 \), ** represent \( P \leq 0.01 \)

The hBD-2 expression can be induced by TNFα above 50 ng/ml and got the optimum induction effect at 150 ng/ml (Fig. 2) in the healthy group and the expression level is up to 16 folds higher than that of the control. In the periodontitis group, the hBD-2 expression can be induced by TNFα at 10 ng/ml and the induced level reached up to 7 times higher than the control (Fig. 2). Significant higher hBD-2 induction was found in healthy subjects at the concentrations of 100, 150 and 200 ng/ml than that in the periodontitis group. While significant higher hBD-2 induction was found in periodontitis patients at the concentration of 10 ng/ml.

The hBD-3 mRNA expression level was up-regulated by TNFα at the concentration of 50 ng/ml in the healthy subjects and also in the periodontitis patients compared with the controls (Fig. 2). Significant higher basal hBD-3 expression level was found in keratinocytes from periodontitis patients. Significant higher hBD-3 inductions were found at the concentration of 10 and 100 ng/ml in periodontitis patients than that of the healthy subject (Fig. 2).

In short, the induction of hBD-1 in keratinocytes from healthy subjects was much easier than that of the patients. The hBD-2 expression can be induced by TNFα at lower dose in keratinocytes from periodontitis patients, while the optimum expression level was much lower. The basal hBD-3 mRNA expression was much higher in keratinocytes from periodontitis patients.

3.3. NF-κB or MAPK pathway played an important role in the induction of hBD-2 and hBD-3

To compare the molecular signalling pathways that are involved in the hBDs induction in healthy subjects and periodontitis patients, we incubated the gingival epithelial cells with MAPK or NF-κB inhibitor before TNFα stimulation. The results showed that there was a significant down-regulation of hBD-1 induction (down to 57%) in keratinocytes from healthy subjects in the presence of MAPK inhibitor (Fig. 3A). While the basal and induced hBD-1 expression was up-regulated in the presence of NF-κB inhibitor and this was beyond our expectations. In keratinocytes from periodontitis patients, the hBD-1 expression was up-regulated by TNFα stimulation and there was a significant down-regulation of induced hBD-1 expression in the presence of MAPK (down to 49%) or NF-κB inhibitor (down to 68%) (Fig. 3B).

There was a reduction in the hBD-2 expression in keratinocytes from healthy subjects at basal level (down to 54%) and subsequent stimulation with MAPK inhibitor (down to 58%). When NF-κB signalling pathway was blocked, the level of hBD-2 induction was reduced to 13% (Fig. 3C). In keratinocytes from
periodontitis patients, the basal hBD-2 expression level was reduced to 63% and induction was reduced to 24% by MAPK inhibitor and 12% by NF-κB inhibitor. The results suggested that both NF-κB and MAPK pathway played important roles in the induction of hBD-2 in keratinocytes from healthy subjects and periodontitis patients (Fig. 3D).

The basal hBD-3 expression level in keratinocytes from healthy subjects was significantly reduced to 54% and the induction was reduced to 40% by MAPK inhibitor. The NF-κB inhibitor reduced hBD-3 mRNA expression to 82% after induction (Fig. 3E). In keratinocytes from periodontitis patients, the basal expression level was not reduced by MAPK inhibitor. The induction of hBD-3 was reduced to 69% by MAPK inhibitor and 45% by NF-κB inhibitor (Fig. 3F). The results also indicated the critical roles of NF-κB and MAPK in TNFα mediated induction of hBD-3.

4. Discussion

The present study showed the different expression levels of human β-defensin-1, -2, and -3 in gingival tissue samples and isolated keratinocytes from chronic periodontitis patients and healthy controls.5-11 The results showed that significantly higher hBD-1, -2 and -3 mRNA expression levels were found in healthy gingival tissues. The results confirmed the results of former studies that higher hBD-2 and hBD-3 expression level were found in healthy gingival tissues than that in periodontitis patients.5,9,11,12 While other studies demonstrated a significantly less frequent expression of hBDs in inflamed oral tissue samples. Similarly, hBD-2 levels in saliva were found diminished in patients with oral candidiasis as compared to healthy controls.18 Consumption of hBDs by local inflammation and variable gene copy numbers in different individuals may be the explanation of this phenomenon. The number of healthy subjects or patients in this study was limited, the expression level of hBDs just showed a higher tendency.

Secondly, primary gingival keratinocyte from chronic periodontitis patients and healthy subjects were utilized in the present study, to evaluate hBDs induction in response to inflammatory mediator TNFα. Many reports have shown an induction of these defensins in cell culture models with various inflammatory mediators and LPS.15,16,19,20 The results suggested that hBD-1 was constitutively expressed whereas hBD-2 and hBD-3 could be induced as a reaction upon infection.20 However, their expression upon induction was highly variable upon different stimuli and was directly correlated with the basal expression levels.15,16,20 To the best of our knowledge, no data is available regarding the comparison of hBDs expression in gingival epithelial cells from periodontitis patients and healthy controls.

In the present study, we incubated the primary keratinocytes with different concentration of TNFα and it was demonstrated that the induction of hBD-1 in keratinocytes from healthy subjects was much easier than that of the patients and this is out of our expectation. hBD-1 was shown constitutively expressed and could not induced in former studies.20 The expression of hBD-2 can be induced by TNFα at lower dose in keratinocytes from periodontitis patients, while the optimum expression level was much lower. That means the cells from periodontitis patients can easily be induced and more prone to recognize and respond to TNFα to express hBD-2. The basal hBD-3 mRNA expression was much higher in keratinocytes from periodontitis patients. However, the hBD-3 expression level in gingival tissue from periodontitis patients was much lower compared with healthy group. The consumption of hBD-3 against chronic periodontitis in local environment may be the cause of reduced expression hBD-3 in the inflamed gingival tissues. It has been suggested that the host response is an important factor in the development of periodontitis.21 That may suggested that hBD-3 played an important role in the local immunological reaction against periodontitis.

Multiple pathways are involved in the induction of hBDs. Earlier studies reported that hBD-2 responses to pathogenic bacteria involved both MAPK and the NF-κB transcription factors.22-27 A recent study of Krisanaprakornkit et al.26 showed that hBD-2 is induced through the MAP-kinase pathway under NF-κB blockade. In our current study utilizing NF-κB and MAPK inhibitors to knockdown interest genes, we provided further evidence that gingival epithelial cells utilize both NF-κB and MAPK signalling pathways in hBD-2 induction in response to TNFα. The epithelial cells have also evolved both pathways to respond to TNFα and express hBD-3. The NF-κB transcription factor pathway is important in the cellular responses to inflammatory stimuli and to the overall response to pathogens in many cell types. Our findings now highlight that in spite of the differences between patients and control subjects at hBDs inductive expression level, the molecular pathways involved in the responses to the inflammatory cytokine were the same. This might be caused by genetic, intrinsic properties of gingival epithelial cells.

In conclusion, the present results demonstrated the that healthy tissue samples showed higher hBD-1, -2 and -3 mRNA expression. The isolated epithelial cells from periodontitis patients respond differently compared with those from healthy subjects when induced by TNFα. The expression of hBD-2 can be induced by TNFα at a lower dose in epithelial cells from periodontitis patients suggest they are more prone to recognize and respond to TNFα. The basal expression of hBD-3 in keratinocytes from periodontitis patients suggested that hBD-3 may play an important role in the immunological reaction against periodontitis. Our study also reinforces the fact that the molecular pathways involved in the responses to the inflammatory cytokine in patients and healthy controls were the same.

Authors’ contribution

Lili Chen and Jiarong Liu conceived and designed the research. Jie Chen, Xingyan Du, and Li Hu performed the research. Jie Chen and Xingyan Du analyzed the data. Jiarong Liu wrote the manuscript.

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The authors do not have any possible competing interest.

Ethical approval

The ethical approval was given by Union Hospital, Tongji Medical college, Huazhong University of Science and Technology, the reference number is S037 (2012).

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