Changes in mRNA Expression of Adenosine Receptors in Human Chronic Periodontitis

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Objective: *To elucidate the aetiology of periodontitis, this study focused on the adenosine receptor (AR) expression profiles (A_1AR, $A_{2A}AR$, $A_{2B}AR$ and A_3AR) in periodontal diseased tissues.*
Methods: *Adenosine receptor gene expression levels in human gingiva from 15 patients with healthy gingival tissues (control group) and 15 patients who exhibited severe chronic periodontitis (test group) were measured using quantitative reverse transcription-polymerase chain reaction (RT-PCR).*
Results: *The mRNA expression pattern changed in human chronic periodontitis: the A_1AR decreased 20%, $A_{2A}AR$ increased 2.5-fold, $A_{2B}AR$ increased 3.7-fold and A_3AR decreased 70% as compared with that of healthy gingiva.*
Conclusion: *Inflammation of the gingival tissue is associated with (1) an unchanged expression of A_1AR, (2) an increased expression of $A_{2A}AR$ and $A_{2B}AR$, and (3) a decreased expression of A_3AR. Logistic regression analysis indicated that the change in the expression patterns can be used to diagnose/predict periodontitis. This finding indicates that the adenosine receptor expression profile is changed in periodontitis with the potential for future clinical application.*

Key words: *adenosine receptor, chronic periodontitis, gene expression profiling, inflammation mediator*

Periodontitis is a chronic disease characterised by inflammatory cell accumulation in the extravascular gingival connective tissue. Inappropriate activation of inflammatory and resident cells becomes self-perpetuating and can lead to chronic periodontal tissue destruction. A shift in microbiological species in gingival sulci from Gram-positive, facultative, fermentative microorganisms to Gram-negative, anaerobic, chemo-organotrophic and photolytic species has been strongly linked to periodontal tissue breakdown[1-3]. However, the molecular mechanisms are still not clear and treatment outcomes are not always satisfactory, making disease prevention more important. Although plaque control is a most important and effective method for the prevention of periodontal disease or its progression, it cannot reverse accumulated tissue damage. Because of this, approaches for disease prevention and early detection are essential.

Adenosine is a signalling molecule that is generated at sites of tissue injury, hypoxia and inflammation. Consistent with this, adenosine levels are elevated in circulation during sepsis[4] and in tissues following ischemia[5]. Studies in animal models revealed that adenosine can have very different regulatory effects in response to inflammation or tissue injury[6-8]. It has potent

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anti-inflammatory and tissue protective effects during acute injury processes such as the ischemia/reperfusion injury[6] or as a response to endotoxin[7]. In contrast, increased levels of adenosine in chronic inflammation and injury models may activate pro-inflammatory and tissue destructive pathways[8]. Such changes indicate that adenosine has a broad range of biological actions in a variety of cells and can modulate the cellular functions affecting the inflammatory responses[9-11]. Moreover, a number of studies have shown that adenosine can promote wound healing through activation of adenosine receptors[12-15], which may be helpful during treatment of chronic periodontitis.

Adenosine, an endogenous nucleoside, is generated intracellularly and extracellularly through dephosphorylation of adenosine monophosphate (AMP) by ecto-5'-nucleotidase (CD73), and metabolised by partial deamination to inosine, mediated by adenosine deaminase, followed by deribosylation and oxidative degradation to uric acid[8,9]. Adenosine activates the cell surface G protein-coupled adenosine receptors (ARs). Four distinct ARs are present in mammalian cells: A_1AR, $A_{2A}AR$, $A_{2B}AR$ and A_3AR^{16}. These receptors have differing affinities for adenosine or its analogs, and their relative levels of expression in cells can vary.

Recently it was shown that adenosine or an adenosine receptor agonist can inhibit *Escherichia coli*[17]. Similarly, adenosine or an adenosine receptor agonist can alter the cytokine production by neutrophils and their adherence to vascular endothelial cells[10,15,18], indicating that adenosine is involved in the interactions of immunocompetent cells with other cell types. Lipopolysaccharide induction of tumour necrosis factor-α production by monocytes or macrophages is well established[19-23]. The suppression of chemokine receptor function, however, appears to involve the adenosine receptor[24]. Furthermore, it was proposed that adenosine receptors could be useful for treatment of infection and sepsis[25,26].

Previous studies have shown that both human gingival epithelial cells and fibroblasts express adenosine receptors, and that their expression may regulate other inflammatory molecules[27-30]. However, the expression profiles of adenosine receptors in diseased periodontal tissues have not been investigated; these might play important roles in disease development, progression and treatment, as indicated in many other diseases. The present null hypothesis was that the adenosine receptor expression profiles are not changed in chronic periodontitis. However, the present data indicate that the expression of adenosine A_{2A} and A_{2B} receptors are increased in human chronic periodontitis, the expres-

sion of the A_3 receptor is decreased, while no significant change is observed in the expression of the A_1 receptor. These findings suggest that adenosine receptors may play important roles in human chronic periodontitis. In addition, these findings should contribute toward better clinical appreciation of the molecular mechanisms involved as well as the possible development of new treatment modalities.

Materials and methods

Site selection

Adult patients, ages 35 to 80 years, with chronic periodontitis treated in the Advanced Education Program in Periodontics at the Loma Linda University School of Dentistry were included in this study. Patients were recruited from June 2008 to September 2008. All patients were systemically healthy. A total of 15 clinically healthy sites and 15 chronically diseased periodontal sites from 15 subjects each were included in this study. The control subjects were recruited from patients requiring periodontal surgery for other reasons, such as crown lengthening or necessary extractions (without periodontitis). The conditions of the periodontal tissues were evaluated by clinical and radiographic examinations. Sites in the clinically healthy (control) group had no colour and texture change to gingival tissue, no visible plaque and calculus, no bleeding upon probing, probing depth (PD) ≤ 3 mm and no radiographic evidence of bone loss. Sites in the periodontitis (test) group had obvious colour and texture change to gingival tissue, PD ≥ 5 mm, clinical attachment loss (CAL) ≥ 5 mm, bleeding upon probing and radiographic evidence of bone loss corresponding to CAL. Patient exclusion criteria were: *(1)* having any uncontrolled systemic disease known to affect periodontal conditions, *(2)* having any conditions for which antibiotic premedication would be required, *(3)* history of antibiotic treatment within the last 3 months and *(4)* history of periodontal treatment within the last 6 months.

Approval for the study was obtained from the Institutional Review Board of Loma Linda University. The patients were asked to sign an informed consent form.

Sample collection and processing

Periodontal tissues were obtained from the junctional epithelium side of periodontal pockets immediately before periodontal treatment, and tissues were processed

Table 1 Primer characteristics

	NCBI number	Positions	Primer sequences	Product lengths
A_1	NM_000674.2	Forward: 441–460 Reverse: 662–643	Forward: 5'- CCTCCATCTCAGCTTTCCAG-3' Reverse: 5'-AGTAGGTCTGTGGCCCAATG-3'	222
A_{2A}	NM_000675.4	Forward: 1389–1408 Reverse: 1619–1600	Forward: 5'- GCAAGAACCTTTCAAGGCAG-3' Reverse: 5'- CTAAGGAGCTCCACGTCTGG-3'	231
A_{2B}	NM_000676.2	Forward: 1560–1579 Reverse: 1764–1745	Forward: 5'- TATGCCAACAGCTTGAATGG-3' Reverse: 5'- TTTTGAGGTCACCTTCCTGG-3'	205
A_3	NM_000677.3	Forward: 1657–1676 Reverse: 1819–1800	Forward: 5'- TCAAAGCTTGTGTGGTCTGC-3' Reverse: 5'- ATGTAAAAATCCCTTGGCCC-3'	163

Primers for ARs were designed using NCBI online software.

for standard RNA extraction and real-time polymerase chain reaction (PCR) measurement as described[31,32]. Briefly, tissue RNAs were stabilised in RNAlater® (Ambion, Austin, TX, USA) then RNAs were extracted using TRI Reagent® (Molecular Research Center, Cincinnati, OH, USA). Total RNA concentrations were quantitated using a NanoDrop™ Spectrophotometer (Thermo Scientific, Waltham, MA, USA), the first strand cDNAs were synthesised using SuperScript III™ First-Strand Synthesis System for reverse transcription-PCR (RT-PCR) (Invitrogen, Carlsbad, CA, USA), and the adenosine receptor expression levels were measured by quantitative RT-PCR using LightCycler® DNA Master SYBR Green I (Roche Applied Science, Indianapolis, IN, USA). All procedures were performed following the respective manufacturer's instructions.

RNA extraction

Periodontal tissues were homogenised by means of a sonic homogeniser in 1 ml Tri Reagent, the homogenate was stored for 5 min at room temperature and then supplemented with 0.2 ml chloroform. The homogenised samples were covered tightly and shaken vigorously for 15 s. The resulting mixture was stored at room temperature for 5 min and centrifuged at 12,000 g for 15 min at 4ºC. The aqueous phase of the mixture, containing RNA, was transferred into a new Eppendorf tube. The RNA was precipitated from the aqueous phase by mixing with 0.5 ml isopropanol. The sample was stored at room temperature for 10 min and centrifuged at 12,000 g for 15 min at 4ºC. The supernatant was removed and the RNA pellet was washed with 1 ml 75% ethanol and subsequently centrifuged at 7,500 g for 5 min at 4ºC. The ethanol was removed and the RNA pellet was air dried briefly at room temperature. RNA was dissolved in 30 µl DEPC-H_2O.

RNA quantitation

RNA concentrations were measured using NanoDrop 1000 with 1 µl of RNA solution each time. The measurements were repeated two to four times.

First-strand cDNA synthesis

Following the manufacturer's instruction, 5 µg RNA was mixed with Oligo (dT) primer and dNTP mix. The mixtures were incubated at 65ºC for 5 min and chilled on ice for at least 1 min. Next, 10 µl cDNA synthesis mix was added to each RNA/primer mix and the samples were incubated at 50ºC for 50 min. The reaction was terminated at 85ºC for 5 min and the samples again chilled on ice. Then, 1µl RNAse H was added to each reaction tube, which were incubated at 37ºC for 20 min. The product was stored at –20ºC for subsequent PCR.

Primer design

Primers for human adenosine receptors were designed by using NCBI online Primer-BLAST software (Table 1) (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=NcbiHomeAd).

Quantitative real-time PCR

Reactions were performed in a 10 µl mix containing 2.5 mM Mg^{2+}, 0.5 µM primer and 1 µl DNA template. Reaction conditions were: 95ºC for 30 s; followed by 95ºC for 0 s, 54ºC for 15 s and 72ºC for 20 s for 45 cycles; followed by 95ºC for 0 s, 65ºC for 15 s and 95ºC for 0 s for one cycle and cooling at 40ºC for 30 s. GAPDH was used as a control.

Table 2 Patient demographics: patient characteristics in both control and test groups are similar

	Test	Control
Age (year, mean)	60 ± 12	59.4 ± 18.2
Age (year, median)	59 (42–78)	58 (24–86)
Gender	7 females	8 females
Diabetes	2 type II	2 type II
Arthritis	3	1
Smoking	1 current smoker	1 former smoker
Hypertension	4	5

Data analysis

The Ct approximation method was used in accordance with the manufacturer's (Roche) instruction to analyse the adenosine receptor expression changes[33]. The changes were expressed as fold change. Fold induction = $2^{-[\Delta\Delta Ct]}$ and $\Delta\Delta Ct$ = [Ct GI (unknown sample) – Ct GAPDH (unknown sample)] – [Ct GI (calibrator sample) – Ct GAPDH (calibrator sample)]. The significance of the expression level change was arbitrarily set at a twofold change. No statistical method was used for expression level change because the expression level change was calculated as fold change.

Descriptive statistics were tabulated as bar graph and standard deviation. Inferential statistics, including *t* test, Pearson's correlation and logistic regression analysis, were performed using SPSS software (version 16.0 for Windows, SPSS, Chicago, IL, USA).

Results

Patient demographics

A total of 30 subjects were enrolled in the study, 15 subjects exhibited severe chronic periodontitis and another 15 patients had a healthy periodontium based on clinical and radiographic diagnosis. Demographic characteristics are summarised in Table 2. The mean age of the test group is 60 ± 12 (42 to 78) years old and for the control group is 59.4 ± 18.2 (24 to 86) years old. Two subjects in each group had controlled type II diabetes. Diabetes of one of the subjects in the test group was controlled by diet and exercise only. The test group had three subjects with arthritis, while there was only one in the control group.

Primer specificity

Primer specificity was determined by conventional RT-PCR. All four primer sets were tested using RNAs from liver cancer, which is known to express adenosine receptors. Each primer set was able to efficiently amplify a single band at the desired size, while the negative control did not yield that band (data not shown).

The expression of adenosine receptors in human gingival tissues, both in healthy and inflamed tissues, was also detected by RT-PCR (data not shown).

Expression levels of adenosine receptors in healthy human gingiva

The expression levels of adenosine receptors in healthy human gingival tissues were determined by crosspoints of real-time RT-PCR. The crosspoint is the number of cycles needed to amplify molecules of interest to a certain predetermined copy number (set by software, Roche Applied Science). Fewer cycles required to reach the crosspoint are associated with higher gene expression.

The cycle numbers to reach crosspoints for A_1AR, $A_{2A}AR$, $A_{2B}AR$ and A_3AR were 25.36, 26.58, 20.96 and 21.27, respectively, in healthy gingiva (Fig 1). These outcomes indicate that $A_{2B}AR$ and A_3AR are expressed at a relatively higher level in healthy human gingival tissue compared with A_1AR and $A_{2A}AR$.

Expression of adenosine receptors in inflamed human gingiva

Changes in the expression of adenosine receptors were measured by real-time RT-PCR, as described above. The expression pattern was altered in human chronic periodontitis. The A_1AR decreased 20%, $A_{2A}AR$ increased
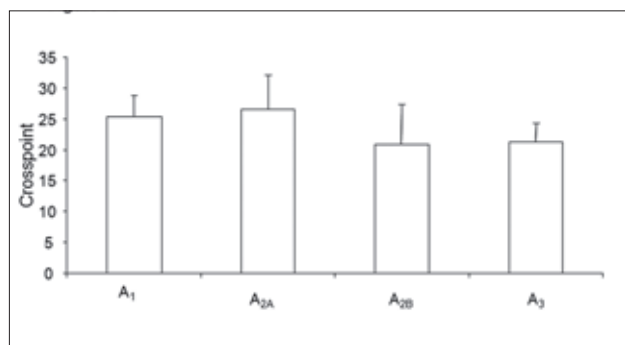
Fig 1 The expression levels of ARs in healthy human gingiva. The expression levels were determined from the number of cycles required to reach crosspoints. The crosspoints were reached in 25.36 ± 3.51, 26.58 ± 5.43, 20.96 ± 6.37 and 21.27 ± 3.09 cycles for A_1AR, $A_{2A}AR$, $A_{2B}AR$ and A_3AR, respectively.
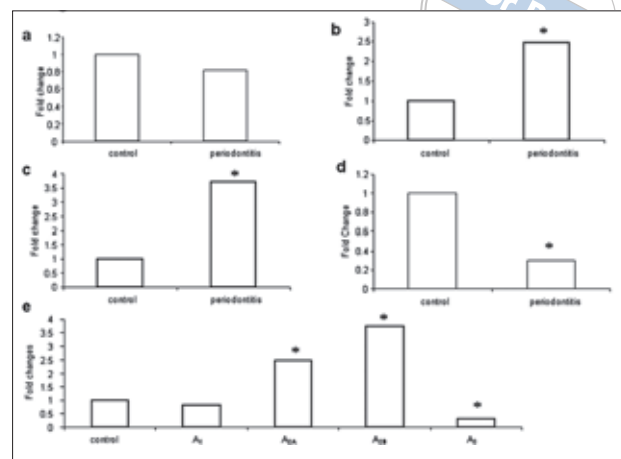

Fig 2 The expression changes of ARs. a) The expression of A_1AR was decreased slightly in human periodontitis tissues. It is at 82% of human healthy gingiva. b) The expression of $A_{2A}AR$ was significantly increased in human periodontitis tissues by 2.5-fold. c)The expression of $A_{2B}AR$ was increased 3.7-fold in human periodontitis tissues. d) The expression of A_3AR was significantly decreased in human periodontitis tissues to 30% of control levels. The expression level in control healthy tissue was designated as 1. e) Combined results from a, b, c, and d. * Designates a two-fold increase or 50% reduction compared to control.

2.5-fold, $A_{2B}AR$ increased 3.7-fold and A_3AR decreased 70%. The change in local expression levels of adenosine A1 receptor was not significant (Figs 2a and 2e). However, local $A_{2A}AR$ and $A_{2B}AR$ expression levels significantly increased (Figs 2b, 2c and 2e), while local A_3AR levels significantly decreased in chronic periodontitis (Figs 2d and 2e).

Predictability of adenosine receptor expression pattern

Logistic regression analysis was used to determine the relationship between the adenosine receptor expression pattern and disease prediction. The results suggest that the expression pattern of adenosine receptors in human chronic periodontitis can be used successfully to diagnose the disease. The expression change of all four adenosine receptors is significantly associated with periodontitis ($P = 0.039$); however, the expression change of either one of these receptors alone is not associated with periodontitis ($P > 0.05$) (Table 3a and 3b). Further analysis using Pearson's correlation test revealed that A_1AR, $A_{2A}AR$ and A_3AR are highly correlated to each other, but $A_{2B}AR$ is not correlated to any other AsAR (Table 3c).

Discussion

Periodontitis is a chronic inflammatory disease, with mild and moderate forms affecting 30% to 50% of adults and the severe form affecting 5% to 15% of total adults in the USA[34]. It is characterised by an accumulation of inflammatory cells in extracellular tissues as well as a shift in microbiological species. The standard initial therapy includes scaling and root planing, but is usually ineffective for certain sites with deep probing depth.

Because virtually all cases of periodontitis present as infectious diseases, they can be prevented or effectively treated by controlling pathogenic microbes residing in subgingival and supragingival plaque. Effective periodontal prevention and therapy are dependent on full-mouth disinfection, such as supragingival debridement, subgingival scaling and root planing, and adequate oral hygiene. Additionally, antibiotic treatments, antiseptic substances and other chemotherapeutic agents can sometimes be effective. Antibiotics, however, may have adverse reactions, such as bacterial resistance, selective effectiveness for certain bacteria and the potential need for customisation. Similarly, most antiseptic agents have a very unpleasant taste[35].
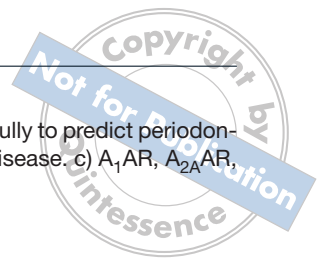
mote wound healing[12-14,37]. The actual role of $A_{2A}AR$ in periodontitis is unknown. The present authors speculate that it may be involved with *(1)* angiogenesis (manifested by increased bleeding on probing), *(2)* suppression of pro-inflammatory host reaction and *(3)* tissue regeneration following active treatment. The A_3AR subtype is perhaps the most enigmatic among adenosine receptors. A recent review stated that several studies attempted to elucidate its physiologic role[38], but it still remains unclear what specific function it is most associated with. However, it appears to be present in a variety of pathophysiologic conditions[38].

Interestingly, the pattern changes in expression levels of ARs can be used to confirm the presence of periodontitis. This may aid earlier detection and diagnosis of periodontitis.

In conclusion, four adenosine receptors were expressed at different levels in human gingival tissues. The expression pattern changed in periodontitis with significant increases of $A_{2A}AR$ and $A_{2B}AR$ and a pronounced decrease of A_3AR. Further studies are needed to elucidate the particular roles of these receptors in periodontitis.

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