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ORIGINAL ARTICLE

Expression of cellular fibronectin mRNA in adult periodontitis and peri-implantitis: a real-time polymerase chain reaction study

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Cellular fibronectin (cFn) is a type of bioactive non-collagen glycoprotein regarded as the main substance used to maintain periodontal attachment. The content of cFn in some specific sites can reflect the progress of periodontitis or peri-implantitis. This study aims to evaluate the expression of cFn messenger RNA (mRNA) in tissues of adult periodontitis and peri-implantitis by real-time fluorescent quantitative polymerase chain reaction (PCR) and to determine its clinical significance. A total of 30 patients were divided into three groups of 10: healthy, adult periodontitis and peri-implantitis. Periodontal tissue biopsies (1 mm×1 mm×1 mm) from each patient were frozen in liquid nitrogen. Total RNA was extracted from these tissues, and the content, purity and integrity were detected. Specific primers were designed according to the sequence, and the mRNA expression levels of cellular fibronectin were detected by real-time PCR. The purity and integrity of the extracted total RNA were both high, and the specificity of amplified genes was very high with no other pollution. The mRNA expression of cFn in the adult periodontitis group (1.526 ± 0.441) was lower than that in the healthy group (3.253 ± 0.736). However, the mRNA expression of cFn in the peri-implantitis group (3.965 ± 0.537) was significantly higher than that in the healthy group. The difference revealed that although both processes were destructive inflammatory reactions in the periodontium, the pathomechanisms were different and the variation started from the transcription level of the *cFn* gene.

INTRODUCTION

Cellular fibronectin (cFn) is a type of non-collagen glycoprotein with an important bioactivity that exists abundantly in the periodontium. Functioning as a substance that maintains the periodontal attachment, cFn takes part in many physiological processes such as cellular adhesion, proliferation and differentiation; restoration and regularization of the epithelium array; and immune regulation. In adult periodontitis, the increment of degraded cFn segments was demonstrated as the result of periodontal tissue inflammation and destruction.[1]

Oral implantation is a breakthrough in the development of dentistry and provides a better choice to restore missing teeth. However, the cause of bone loss and failure of implantation in peri-implantitis needs to be explored. The content of cFn in periodontal tissue may be related to the state of implants because it is a vital component of the extracellular matrix.[2]

So far, studies on the expression of cFn in the periodontium are not sufficient. Experiments at the protein level have been performed through immunohistochemistry,[3] and the expression of cFn messenger RNA (mRNA) in the periodontium has been examined using reverse transcription polymerase chain reaction (PCR).[4] However, the immunohistochemistry test is merely qualitative, has high subjectivity, and sometimes results in certain false positive and negative rates. Reverse transcription PCR could be used to detect minute mRNA, but its doubtful accuracy and tedious steps may not result in a convincing outcome.

Real-time fluorescent quantitative PCR could precisely solve the above problems. By adding fluorescent radical in the reaction system, the whole amplificative process could be monitored through the cumulative fluorescent signal. After working out the standard curve and obtaining the cycle threshold (C_t) value of one sample, the content of the amplified gene could be directly measured through the curve.

This study aims to observe the expression discrepancy of cFn mRNA in patients with periodontitis and peri-implantitis at the transcriptional level by real-time PCR, and discuss the relation between cFn and the development of these diseases.

MATERIAL AND METHODS

Subjects and sample collection

A total of 30 subjects referred to the Out-patient Department of West China Hospital of Stomatology, Sichuan University were divided into three groups of 10: periodontitis, peri-implantitis and healthy control. Inflamed gingival tissues (approximately 1 mm×1 mm×1 mm) were collected from the active periodontal inflammation site of 10 chronic periodontitis subjects (five males and five females aged 20–60 years

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with an average age of 45.0 years) under the following inclusion criteria: gingival index >1, at least five sites with probing depth ≥4 mm, clinical attachment loss ≥1–2 mm and extensive radiographic bone loss ≥1/3 of the root length in the surgery quadrant while sampling. Gingival tissues (approximately 1 mm×1 mm×1 mm) of the peri-implantitis patients were collected from the obvious inflammation site in 10 subjects (five males and five females aged 20–60 years with an average age of 47.4 years) under the following inclusion criteria: pain and swelling signs in gingiva with/without presence of pus, movable parakeratosis mucosa around the implant body, and radiographic examination showing marginal bone loss over three or more fixture threads mesially or distally. Healthy gingival tissues (approximately 1 mm×1 mm×1 mm) were collected from the normal periodontium of 10 healthy subjects (five males and five females aged 20–60 years with an average age of 44.8 years) after teeth extraction for orthodontic or impacted reason under the following inclusion criteria: probing depth <3 mm, clinical attachment loss <1 mm and without radiographic evidence of alveolar bone loss in the whole dentition. The samples from all subjects were acquired through periodontium biopsy. Exclusion criteria were as follows: individuals with known systemic risk factors such as diabetes or smoking, patients on medications, pregnant women and immunoincompetent subjects. Age and gender were matched in three groups. The purpose and procedures of the study were explained to the subjects, and informed consent was obtained from each individual. The protocol of this study was approved by the ethics committee of the West China Hospital of Stomatology, Sichuan University.

Pre-treatment of samples

The specimens were immediately washed with saline solution and stored in liquid nitrogen (YDS-3, Jinfeng Liquid Nitrogen Co. Ltd., Chengdu, China) and then transferred into a refrigerator at −80 ℃ (Ultra-Low Temperature Fridge; NuAire Inc., Plymouth, USA).

Tissue masses (50 mg) acquired from each specimen were cut into pieces and transferred into a 1.5 mL Eppendorf (EP) tube containing 1 mL Trizol. The homogenate was immediately crushed. The EP tube was placed on ice for a while and then centrifuged (12 000 r·min^{-1}) at 4 ℃ (Megafuge 1.0R microthermal high-speed centrifuge; Heraeus, Hanau, Germany). The supernatant was shifted into an EP tube pre-treated with di-ethylpyrocarbonate. Finally, the sample was placed at room temperature for 5 min to crack completely.

RNA extraction

After adding 0.2 mL chloroform into the pre-treated samples, they were placed in a spiral whirling device for 15 s and allowed to stand at room temperature for 2–3 min. Afterwards, the samples were centrifuged at 12 000 r·min^{-1} for 15 min at 4 ℃. The supernatant was transferred into an EP tube pre-treated with di-ethylpyrocarbonate. The EP tube was added with 0.5 mL isopropanol, turned upside down, and then placed on ice for 10 min of precipitation. The sample was centrifuged at 12 000 r·min^{-1} for 10 min at 4 ℃. A gelatinous RNA deposit was observed at the bottom of the tube; the supernatant was discarded. After adding 1 mL of 75% ethanol and mixing in a spiral whirling device, the sample was centrifuged again at 12 000 r·min^{-1} for 5 min at 4 ℃. The supernatant was then discarded. The liquid was completely absorbed after further instant centrifugation. Finally, the RNA was dried, uncovered, dissolved in 20 μL di-ethylpyrocarbonate and then preserved at −80 ℃.

RNA quality testing

The ratio of OD_{260}/OD_{280} after diluting the RNA solution was measured, and electrophoresis with a 5 μL sample in 1% agarose gel (electrophoresis apparatus; Bio-Rad Inc., Hercules, CA, USA) was performed to detect the purity of total RNA.

Reverse transcription

Using the mRNA in the total RNA as template, cDNA was obtained by reverse transcription with a random primer using a Revert Aid First Strand cDNA Synthesis Kit (MBI Inc., Davis, CA, USA). Total RNA (5 μL) was premixed with 1 μL of 0.2 μg·μL^{-1} random hexamer primer and 6 μL of nuclease-free deionized water. Instant centrifugation was then performed, followed by pre-treatment at 70 ℃ for 5 min, and then ice cooling. Approximately 4 μL of 5× reaction buffer, 1 μL of 20 U·μL^{-1} recombinant ribonuclease inhibitor, 2 μL of 10 mmol·L^{-1} dNTP mix and 1 μL of 200 U·μL^{-1} RevertAid M-MuLV reverse transcriptase were added into the mixture, followed by centrifugation. The reaction was performed at 20 ℃ for 10 min, 42 ℃ for 60 min, followed by 70 ℃ for 10 min in a PCR instrument (PerkinElmer Genetics, Bridgeville, PA, USA). Finally, the acquired cDNA solutions were preserved in a refrigerator at −20 ℃.

Design of primer

According to the gene sequence of cFn in GenBank (Gene ID: 2335) and the primer designing rules, the upstream and downstream PCR primers for cFn were designed as 5′-CCA TCG CAA ACC GCT GCC AT-3′ and 5′-AAC ACT TCT CAG CTA TGG GCT T-3′, respectively. In this experiment, the gene of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was selected as reference. According to its sequence in GenBank (Gene ID: NM_002046), the sequences of the upstream and downstream PCR primers for GAPDH were 5′-AAG AAG GTG GTG AAG CAG GC-3′ and 5′-TCC ACC ACC CTG TTG CTG TA-3′, respectively.

Processing real-time PCR

We diluted the cDNA solution based on a specific gradient (10^0, 10^1, 10^2, 10^3, 10^4 and 10^5) with EASY dilution. Each reaction contained 3 μL of Mg^{2+} free 10× buffer, 3 μL of 25 mmol·L^{-1} MgCl$_2$, 0.36 μL of 25 mmol·L^{-1} dNTP, 1 μL of 10 μmol·L^{-1} upstream primer, 1 μL of 10 μmol·L^{-1} downstream primer, 1 μL of 10 μmol·L^{-1} 10 000× SYBR green I, 0.3 μL of 5 U·μL^{-1} Taq polymerase, 15.34 μL of ddH$_2$O and 5 μL of cDNA template. Amplification was performed by 45 cycles of 94 ℃ for 20 s, 55 ℃ for 20 s, 72 ℃ for 30 s and 80 ℃ for 20 s in an FTC2000 fluorescent real-time PCR instrument (Funglyn Biotech Co., Scarborough, Canada). The target gene and reference gene were amplified. After amplification, the whole system was heated slowly to 95 ℃ (increasing by 1 ℃ every 5 s) to obtain the melting curve.

Statistical analysis

Statistical calculations were made using a software package (SPSS 17.0; IBM Co., New York, NY, USA). The differences were considered significant when the probability value was less than 5% ($P<0.05$). SKEW and KURT tests were first used to test the data for normality. Levene test was then conducted to examine the homogeneity of variance for multiple samples.

Normally distributed data were expressed as means and standard deviations. For three-group comparisons, one-way analysis of variance was adopted first, aiming to examine whether the differences exist among these three groups, that is, whether the effect of random error (variation within groups, caused by age, gender, *etc.*) could be neglected comparing with the effect caused by grouping factor(peiodontitis, peri-implantitis, healthy). If the differences indeed exist among these groups, then Dunnett's *t*-test would be applied to
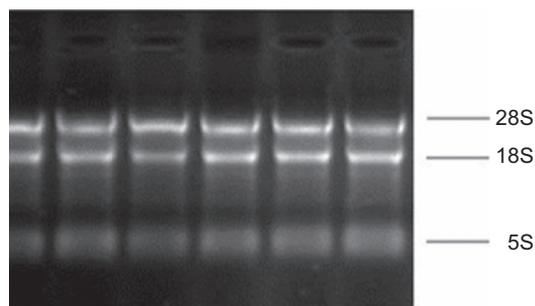
Figure 1 Partial electrophoresis pattern of total RNA for all the samples. Different lanes correspond to samples extracted from different subjects. The brightness of the three bands (28S rRNA, 18S rRNA and 5S rRNA) was degressive, and no obvious dispersive trace could be found. The ratio of brightness for 28S and 18S was around 2:1, illustrating that the RNA is of high quality. rRNA, ribosomal RNA.

evaluate the difference between any test group and the control group.

RESULTS

### Evaluation of the purity and integrity of total RNA
The OD_{260}/OD_{280} of all samples' RNA was between 1.8 and 2.0, indicating that the purity of total RNA was relatively high. The bands of 28S, 18S and 5S from the electrophoresis were distinct and showed no degraded bands (Figure 1).

The melting curve
As shown in Figure 2, the melting curve of the PCR products indicated that only one single temperature peak appeared for the cFn- and GAPDH-amplifying segments. This result indicates that the specificity
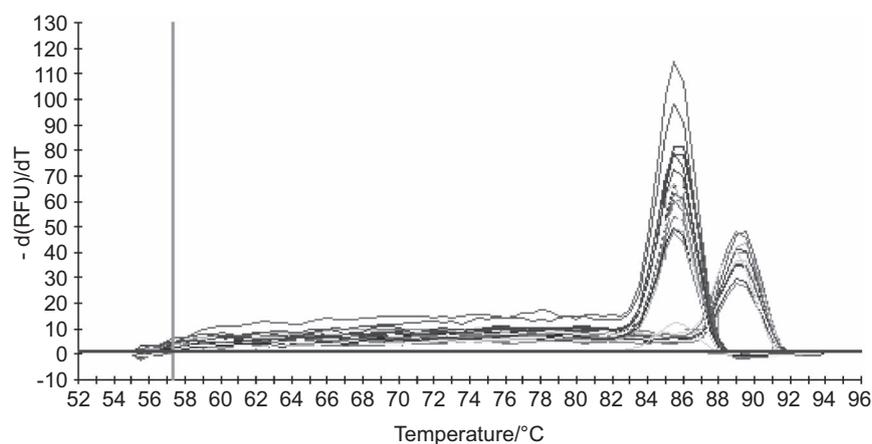
Figure 2 Melting curve of PCR products. The abscissa represents temperature, whereas the ordinate stands for change in fluorescent intensity. A single peak for GAPDH- (left) and cFn- (right) amplified products shows the high specificity of the primer. cFn, cellular fibronectin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PCR, polymerase chain reaction.
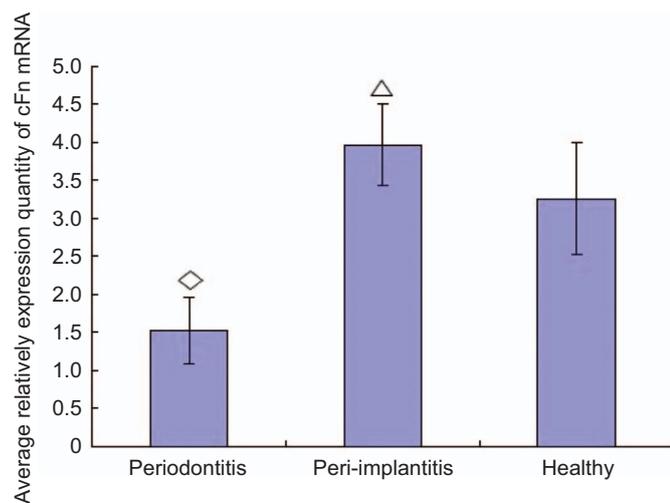
Figure 3 Comparison of average relative quantity for cFn mRNA among the periodontitis, peri-implantitis and healthy groups. Values and error bars of average relative expression quantity represent the means and s.d. of three groups ($n = 10$). $^{\diamond}P < 0.01$ *versus* healthy controls; $^{\triangle}P < 0.05$ *versus* healthy controls. cFn, cellular fibronectin; mRNA, messenger RNA.

of both segments was very high and that no other pollution from the non-specific strips was observed.

mRNA expression of cFn in the periodontitis, peri-implantitis and healthy groups
The cFn mRNA could be detected in all the samples acquired from the periodontitis, peri-implantitis and healthy groups. The average relative expression quantities of cFn mRNA in the periodontitis, peri-implantitis and healthy groups were 1.526 ± 0.441, 3.965 ± 0.537 and 3.253 ± 0.736, respectively. As shown in Figure 3, this result indicates that the transcription of cFn in the periodontitis group was lower than that in the healthy group ($t_D = -6.613$, $P < 0.01$), while the mRNA expression of cFn in the peri-implantitis group was higher than that in the healthy group ($t_D = 2.732$, $P < 0.05$).

DISCUSSION
Fibronectin has two major forms: plasma fibronectin and cFn. Plasma fibronectin, the product of hepatocytes and endothelial cells, is soluble and exists in plasma and body fluids, where it stimulates blood clot formation[5] and wound healing.[6] cFn is secreted by a series of cells, mainly fibroblasts, in an insoluble form and is found in the extracellular matrix or on the surface of cells.[7–9] cFn is a major non-collagenous

glycoprotein with multiple adhesive properties in periodontal tissues, functioning as a vital link between cells and their extracellular matrix.[10]

The concentration of cFn in gingival crevicular fluid was found to be higher in healthy sites than in gingivitis sites.[11] The degradation of cFn tends to be greater at diseased sites than at healthy or treated sites of periodontitis.[12] Mintz and Fives-Taylor[13] discovered that the level of cFn in individuals with adult periodontitis is significantly lower than that in individuals with healthy periodontal condition. Ma et al.[14–15] reported that the quantity of periodontal cFn is higher in patients with peri-implantitis than in healthy individuals.

In connection with the decreasing cFn level in periodontitis, human neutrophil elastase has an important function. Neutrophil elastase is secreted by neutrophilic leukocytes, which are attracted to infected periodontal tissues by chemoattractants from bacteria, host cells or degraded tissue.[16–17] Neutrophil elastase is the most important enzyme that degrades fibronectin to Fn fragments in vivo.[18–19] The activity of neutrophils to release elastase is significantly increased in periodontitis patients than in healthy controls.[20–22] An increase in neutrophil elastase activity increases fibronectin degradation fragments and decreases periodontal mechanical strength.[23] In addition, clinical treatments such as scaling, root planning and curettage weaken elastase activity and restore cFn level.[24]

However, the situation differs in patients with peri-implantitis. In fibroblast cell culture, titanium (Ti) can significantly enhance the expression of cFn.[3] Ti surfaces have an affinity for fibronectin, which enables cFn to bind to the implant surface.[25–26] Various studies have demonstrated that implant sites with peri-implantitis have higher elastase activity than healthy sites.[27–28] However, the expression of cFn is still higher in patients with peri-implantitis than in individuals with healthy periodontal condition. Thus, the generation of cFn induced by Ti implants surpasses its degradation by elastase.

In this study, we compared the mRNA expression of cFn in periodontitis, peri-implantitis and healthy gingival tissues. Real-time PCR results displayed that the transcription of cFn in the peri-implantitis group was higher than that in the healthy group. By contrast, the mRNA expression of cFn in the periodontitis group was lower than that in the healthy group. This result suggests that in periodontitis, the decrease in cFn is not only due to the rising elastase level, but also to the declining mRNA quantity, which may lead to a more decisive effect. Thus, the inflammation process may affect the gene expression of periodontal cells, and cFn may act as a marker that indicates the impairment condition or therapy effect in periodontitis. However, in cases with peri-implantitis, the gene transcription intensifies regardless of the inflammation situation. We speculate that Ti implants stimulate cFn gene transcription and that this incitement offsets the effect of inflammation on periodontal cells. The elevated transcriptional level of cFn may be attributed to ability of Ti to enhance the fibroblast attachment for implants in vivo. The level of cFn is slightly related to the process of inflammation in peri-implantitis. Thus, cFn quantity may not serve as an index in this disease.

The real-time PCR technique utilized in this experiment was designed according to the reaction kinetics of PCR. With the fluorescent dye SYBR Green I, the standard curve of the cFn and GAPDH genes could be drawn according to the dilution degree and their C_t value. As for the standard curve, the concentration of the target gene could be calculated if the C_t value was obtained. Then, the content of the cFn gene was divided by that of the reference gene in the same sample. This way, the comparison for the expression level of all target genes would be on the same base, and the results would be credible and repeatable. The average relative expression quantities of mRNA in the results were acquired in this method.

CONCLUSIONS

The mRNA expression of cFn in the periodontitis group was lower than that in the healthy group. By contrast, the mRNA expression of cFn in the peri-implantitis group was higher than that in the healthy group. This change began during the transcriptional period. Although these two diseases are both inflammatory destructive processes in the periodontium, the pathomechanisms are somewhat diverse. cFn may be recognized as an indicator of periodontitis but not of peri-implantitis because Ti may affect the expression of cFn in the periodontium.

However, due to the small sample size and the different nature of the samples collected, bias may have been introduced. Further experiments are expected to support the conclusions above and to investigate the functions of other substance in these two diseases.

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