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Increased and Correlated Expression of CTGF and TGF β 1 in Surgically Removed Periodontal Tissues with Chronic Periodontitis

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Abstract

Background and Objective—Both gingival tissue destruction and regeneration are associated with chronic periodontitis although the former overwhelms the latter. Studies have shown that transforming growth factor beta (TGF β)1, a growth factor largely involved in tissue regeneration and remodeling, is upregulated in chronic periodontitis. However, the gingival expression of connective tissue growth factor (CTGF or CCN2), a TGF β 1-upregulated gene, in patients with periodontitis remains undetermined. Although both CTGF/CCN2 and TGF β 1 increase extracellular matrix, they have many different biological functions. Therefore, it is important to delineate the impact of periodontitis on gingival CTGF/CCN2 expression.

Material and Methods—Periodontal tissue specimens were collected from 7 individuals without periodontitis (group 1) and 14 with periodontitis (group 2). The CTGF and TGF β 1 mRNA were quantified using real-time polymerase chain reaction.

Results—Analysis using the nonparametric Mann-Whitney test showed that both CTGF/CCN2 and TGF β 1 mRNA expression levels were significantly increased in individuals with periodontitis as compared to individuals without periodontitis. Further, analysis using the nonparametric correlation (Spearman r) test showed a positive correlation between TGF β 1 and CTGF/CCN2 mRNA.

Conclusion—The gingival CTGF/CCN2 and TGF β 1 mRNA expression in individuals with periodontitis are upregulated and correlated.

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Keywords

Periodontitis; growth factors; CTGF; TGF β 1; gene expression

Connective tissue growth factor (CTGF), also called CCN2, is a member of the CYR61, CTGF and NOV (CCN) family and plays an important role in cell proliferation, migration, angiogenesis, wound healing, and matrix production, and is critically involved in fibrotic diseases (1, 2). Studies have shown that the expression of CCN proteins including CTGF/CCN2 is increased in diseases related to chronic inflammation such as atherosclerosis, arthritis, cancers, and diabetic nephropathy and retinopathy (1). CTGF/CCN2 is produced by a variety of cells including monocytes, macrophages, fibroblasts, and epithelial cells and upregulated by transforming growth factor beta (TGF β), inflammatory cytokines, high glucose and environment changes such as hypoxia and biomechanical stimuli (3).

TGF β and CTGF/CCN2 have pleiotrophic properties that play an important role in connective tissue homeostasis (3). It is well known that TGF β and CTGF/CCN2 stimulate tissue remodeling and wound healing by increasing fibroblast proliferation, angiogenesis, and extracellular matrix (ECM) production (1). In addition, TGF β is also a potent inhibitor of matrix metalloproteins (MMPs) and thus reduces collagen degradation (4). Paradoxically, studies have shown that TGF β and CTGF/CCN2 also promote inflammation-associated tissue degradation. For example, Jagels et al. reported that TGF β enhances production of granulocyte-macrophage colony-stimulating factor (GM-CSF) and regulated upon activation, normal T cell expressed and secreted (RANTES) and thus increases recruitment of eosinophils, lymphocytes and monocytes that are involved in chronic and allergic inflammation and tissue destruction (5). Nozawa et al. also reported that CTGF/CCN2 synergizes with macrophage colony-stimulating factor (M-CSF) and receptor activator of NF κ B ligand (RANKL) to enhance osteoclastogenesis and thus contributes to bone destruction in rheumatoid arthritis (6). Apparently, while TGF β and CTGF/CCN2 primarily stimulate connective tissue production and promote fibrosis, they may have complex effects on both tissue regeneration and destruction in the setting of inflammation.

Chronic periodontitis is triggered by Gram-negative bacteria and characterized by inflammatory tissue destruction of tooth-supporting structures of the periodontium. Interestingly, Lorencini et al. reported that gingival collagen fibers were reduced by 30%, but the fibers were thicker and more densely packed in rats with ligature-induced periodontitis when compared with rats without periodontitis, indicating that tissue inflammation and destruction are accompanied by tissue regeneration and remodeling in periodontitis (7). In consistence with this notion, studies have shown that periodontitis is associated with increased expression of TGF β (8, 9), which may play a role in gingival tissue remodeling. However, the periodontal CTGF/CCN2 expression in patients with periodontitis remains unknown although it has been shown that CTGF/CCN2 is co-expressed with TGF β in the gingival mucosa (10).

In this study, we collected periodontal tissue specimens from 21 individuals with or without periodontitis at the time of indicated surgical procedures and quantified CTGF/CCN2 and TGF β 1 mRNA by real-time polymerase chain reaction (PCR). Results showed that the

expression of both CTGF/CCN2 and TGF β 1 mRNA was significantly increased in patients with periodontitis.

MATERIAL & METHODS

Patients

Twenty-one participants including 7 without periodontitis (group 1) and 14 with periodontitis (group 2) were included in this study. Participants in group 1 who had oral surgeries such as crown lengthening, extractions, and periodontal plastic surgery served as controls. All the tissue specimens collected were periodontal tissues. Fourteen individuals in groups 2 met the diagnostic criteria for chronic periodontitis: periodontal pocket depth (PPD) $\geq$ 6 mm in 2 or more teeth or clinical attachment loss (CAL) $\geq$ 5 mm in 2 or more teeth. Radiographic evidence of alveolar bone loss was apparent. The oral examination was conducted as previously described (11). The exclusion criteria were: serum creatinine $\geq$ 1.6 mg/dl, abnormal hepatic function, hemoglobinopathy, unwillingness to sign the informed consent form or enter the study, aggressive periodontitis, and any platelet and coagulation disorders. Individuals in groups 2 had periodontal surgery and specimens were obtained from the greatest PPD and/or CAL sites. All participants provided informed consents for the specimen collection. The study protocol and consent form were approved by the Medical University of South Carolina Institutional Review Board.

Isolation of RNA and RNA Reverse Transcription

Total RNA was isolated from periodontal tissue specimens using the RNeasy minikit (Qiagen, Santa Clarita, CA). The first-strand complementary DNA (cDNA) was synthesized with the iScriptTM cDNA synthesis kit (Bio-Rad, Hercules, CA) by following the instruction provided by the manufacturer. The reaction was cycled for 5 minutes at 25°C, 30 minutes at 42°C and 5 minutes at 85°C using a PTC-200 DNA Engine (MJ Research, Waltham, MA).

Quantitative Real-Time PCR

The reverse transcription reaction mixture from the above experiments was then subjected to PCR amplification. The Beacon Designer Software (PREMIER Biosoft International, Palo Alto, CA) was used for designing primers of CTGF/CCN2 and TGF β 1 (Table 2). Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). PCR was carried out in duplicates using a 25 μ l reaction mixture that contained 1.5 μ l RT reaction mixture, 0.2 μ M of both primers and 12.5 μ l of iQTM SYBR Green Supermix (Bio-Rad). The PCR reaction was performed with the iCyclerTM Real-Time Detection System (Bio-Rad). Forty cycles consisting of denaturation (95°C for 10 sec) and annealing/extension (56°C for 45 sec) were run. A melt-curve experiment was subsequently performed (55°C for 1 min and then temperature is increased by 0.5°C every 10 sec) to detect the primer dimers and verify the specific amplification. Data were analyzed with the SmartCycler II software. The average threshold cycle (*C_t*) of fluorescent units was used for analysis. Quantification was calculated using the *C_t* of the target signal relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signal in the same RNA sample.

Statistical Analysis

The age, gender and race of the study participants were presented as counts and the continuous variables of CTGF/CCN2 and TGFβ1 mRNA were presented as medians. The GraphPad Instat 3 software (GraphPad Software, Inc., San Diego, CA) was used for statistical analysis. Nonparametric analyses test using the Mann-Whitney procedure were performed to detect significant differences in continuous variable between the two groups based on the two-tailed *p* value. Nonparametric correlation analyses using Spearman's rank correlation coefficient were performed to determine if CTGF/CCN2 was correlated with TGFβ1 mRNA expression. A value of *p*<0.05 was considered significant.

RESULTS

Study Population

Demographic and clinical data including patients' age, gender, race and status of periodontitis are presented in Table 2. The ages in groups 1 and 2 were similar (*p*>0.05), ranging from 41 to 74 and 43 to 76 years with mean ± SD of 65 ± 10 and 63 ± 9, respectively. The male/female ratios in groups 1 and 2 were 3/4 and 8/6, respectively. *Race*: All patients recruited were either Whites or non-Hispanic Blacks. The White/Black ratios in groups 1 and 2 were 6/1 and 10/4, respectively. The PPD and CAL between group 1 and group 2 were significantly different (PPD: 2.86 ± 0.34 vs. 6.9 ± 1.5, *p*<0.001; CAL: 2.71 ± 0.69 vs. 7.4 ± 2.3, *p*<0.001).

Periodontal Expression of CTGF/CCN2 and TGFβ1 mRNA in Participants with or without Chronic Periodontitis

Expression of CTGF/CCN2 and TGFβ1 mRNA in periodontal tissue specimens was successfully quantified using real-time PCR. The specific amplification of CTGF/CCN2, TGFβ1 and GAPDH cDNA was confirmed by the melt-curve analysis that showed a single peak for each gene. Results showed that the TGFβ1 mRNA expression is much higher than CTGF/CCN2 expression (Table 3). Furthermore, CTGF/CCN2 mRNA levels had a much larger variation than that of TGFβ1 expression. Statistical analysis using nonparametric Mann-Whitney test showed that both CTGF/CCN2 and TGFβ1 mRNA expression levels were significantly increased in participants with periodontitis when compared to those who did not have periodontitis (*p*<0.01) (Table 3).

Correlation between TGFβ1 and CTGF/CCN2 mRNA expression in participants with periodontitis

To reveal the relationship between TGFβ1 and CTGF/CCN2 mRNA expression in periodontal tissues specimens of participants with periodontitis, we performed a correlation analysis. Using nonparametric correlation (Spearman *r*) test, our analysis showed a positive correlation between TGFβ1 and CTGF/CCN2 mRNA expression (*r* = 0.582; *p* = 0.029) (Fig. 1).

DISCUSSION

It has been reported that the TGF β 1 protein level in gingival tissues and gingival crevicular fluid (GCF) samples collected from the sites with deeper periodontal pockets is higher than that from less periodontally involved sites (8). In addition, the TGF β 1 mRNA level was also found to be increased in gingival tissue from patients with periodontitis when compared to that from individuals without periodontitis (12). Surprisingly, to date there is a lack of the studies on the periodontal expression of CTGF/CCN2, a TGF β 1-regulated gene, in patients with periodontitis. In our current study, we have successfully quantified periodontal mRNA expression of CTGF/CCN2 along with TGF β 1 in both individuals without periodontitis and participants with periodontitis, and reported for the first time that CTGF/CCN2 and TGF β 1 mRNA expression in patients with periodontitis are increased and correlated.

Our current knowledge on the pathogenesis of chronic periodontitis has accentuated the interaction between periodontopathic bacteria and the host immune system that results in a chronic periodontal inflammation and ultimate destruction of supporting tissue and consequently, tooth loss (13). While a great effort has been made to illustrate gingival inflammation and destruction during the progression of periodontitis, studies on tissue regeneration and remodeling in periodontitis are lacking. It is likely that both tissue destruction and tissue regeneration co-exist in periodontitis, although the former overwhelms the latter. In fact, one of the main goals of periodontal therapy is to recreate a balance between tissue destruction and tissue regeneration by reducing tissue inflammation. Interestingly, Spoto and coworkers reported that a significant decrease in GCF alkaline phosphatase (ALP) activity, which is associated with periodontal tissue destruction, was observed 15 days after scaling and root planning (SRP) in patients with periodontitis and the decrease was corresponded to a reduction in the clinical signs of inflammation (14). They further found that the GCF ALP activity was increased 60 days after SRP and the increase was correlated to a subclinical recurrent inflammation.

Inflammatory cytokines play pivotal roles in not only the tissue destruction, but also tissue regeneration (15). It has been reported that while IL-1 and TNF α inhibit collagen biosynthesis during the fracture healing-associated inflammation, they also initiate the tissue repair process and play important roles in intramembraneous and trabecular bone formation (16). Indeed, recent studies have described a paradoxical scenario in which certain pro-inflammatory mediators are not only responsible for periodontal tissue inflammation and destruction, but also required for bone healing (17). Surprisingly, it has been shown that depletion of peripheral blood monocytes, the precursors of tissue macrophages that play a pivotal role in tissue inflammation and destruction, resulted in a marked delay in normal fibroblast proliferation and wound healing (18, 19). To further understand how inflammatory cytokines initiate tissue regeneration, studies have shown that cytokines such as TNF α induces TGF β 1 expression in lung fibroblasts (20), and TGF β 1 is well known for its role in collagen production and tissue remodeling (21). The findings from our current study that periodontal expression of both CTGF/CCN2 and TGF β 1 mRNA was increased in patients with periodontitis support the above notion that both tissue destruction and regeneration are involved in chronic inflammatory diseases. In addition to the increased expression of CTGF/CCN2 and TGF β 1 mRNA, we also demonstrated that the increased

expression of CTGF/CCN2 mRNA was correlated with that of TGF β 1 mRNA in patients with periodontitis. This finding is consistent with the observation by Takeuchi et al. in their study that demonstrated the upregulation of CTGF/CCN2 mRNA and protein expression in human gingival fibroblasts and periodontal ligament cells by TGF β 1 in vitro (22).

The crucial role of TGF β 1 and CTGF/CCN2 in drug-induced gingival overgrowth has been well documented (23). Antiepileptic drugs, calcium channel blockers and immunosuppressant drugs are the major medications that induce gingival overgrowth. To elucidate the pathogenesis of drug-induced gingival overgrowth, studies have shown that cyclosporine stimulates proliferation of gingival fibroblasts and synthesis of collagen via TGF β 1 (24, 25). As a downstream target of TGF β 1, CTGF/CCN2 is also significantly expressed in gingival fibroblasts and contribute to tissue repairing and remodeling in drug-induced gingival overgrowth (26). Studies have shown that the main histologic features of gingival overgrowth are accumulation of extracellular matrix, proliferation of fibroblasts and epithelial cells and various levels of inflammation (27). Obviously, tissue regenerative process is dominant in drug-induced gingival overgrowth (23), which is sharply contrasted to the pathological features of periodontitis in which gingival tissue inflammation and destruction are predominant.

In conclusion, our current study demonstrated that the expression of TGF β 1 and CTGF/CCN2 mRNA in surgically removed periodontal tissues of individuals with periodontitis was increased when compared with that of participants without periodontitis. Our study also showed that the expression of TGF β 1 mRNA was correlated with that of CTGF/CCN2 mRNA.

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Figure 1.

The correlation between CTGF/CCN2 and TGFβ1 expression in periodontal tissues of patients with chronic periodontitis was studied using nonparametric correlation analysis with Spearman's rank correlation coefficient. Both CTGF/CCN2 and TGFβ1 expressions were normalized to GAPDH expression for the analysis.

Table 1

Characteristics of Participants

	Group 1 (patients without periodontitis)	Group 2 (patients with periodontitis)
Number of patients	7	14
Age	65 ± 10	63 ± 9
Gender (male/female)	3/4	8/6
Race	6	10
White	1	4
Black		
Periodontal pocket depth (PPD)	2.86 ± 0.34	6.9 ± 1.5
Clinical attachment loss (CAL)	2.71 ± 0.69	7.4 ± 2.3

The data presented are mean ± SD.

Table 2

The Primer Sequences for Real-Time PCR

Genes	5' primer sequence	3' primer sequence
CTGF/CCN2	CTGGGAAGCCATCACTTACCTTGC	GTTTCTAGAGTCGCTGGGAAGCTG
TGFβ1	AACGCACTAACTTGACCTACAG	CTCCAGAGTTCAAAGGCATCC
GAPDH	GAATTTGGCTACAGCAACAGGGTG	TCTCTTCCTCTTGTGCTCTTGCTG

Table 3Periodontal Expression of CTGF/CCN2 and TGF β 1 in Participants with or without Chronic Periodontitis

	Group 1 (Without periodontitis, n = 7)	Group 2 (With periodontitis, n = 14)	Nonparametric analysis testing for any differences in groups (Mann-Whitney Test)	
			U-statistic	p value
Median of the ratios between CTGF/CCN2 and GAPDH	2.57×10^{-8}	1.7×10^{-3}	9.00	0.002
Median of the ratios between TGF β 1 and GAPDH	1.03	2.84	12.00	0.004