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Short communication

Expression of suppressors of cytokine signaling in diseased periodontal tissues: a stop signal for disease progression?

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Background and Objective: Inflammatory cytokines are thought to trigger periodontal tissue destruction. In addition to being regulated by anti-inflammatory mediators, their activity is under the control of suppressors of cytokine signaling (SOCS), which down-regulate the signal transduction as part of an inhibitory feedback loop. We therefore investigated the expression of SOCS-1, -2 and -3, and the cytokines tumor necrosis factor-α (TNF-α) and interleukin-10, in different forms of human periodontal diseases.

Material and Methods: Quantitative polymerase chain reaction (RealTime-PCR) was performed with mRNA from gingival biopsies of control subjects and from that of patients with chronic gingivitis and chronic periodontitis.

Results: Our results show that patients with chronic gingivitis and chronic periodontitis exhibit significantly higher SOCS-1, -2 and -3, TNF-α and interleukin-10 mRNA expression when compared with healthy controls. The data also demonstrate that SOCS-1 and -3 mRNA expression was higher in tissue from patients with chronic gingivitis than chronic periodontitis, while the levels of SOCS-2, TNF-α and interleukin-10 mRNA were similar in these groups.

Conclusion: The increased expression of SOCS-1, -2 and -3 mRNA in diseased periodontal tissues is believed to be involved in the down-regulation of inflammatory cytokine and Toll-like receptor signaling, and therefore in the attenuation of both the inflammatory reaction and disease severity. Furthermore, it is possible that variation in the levels of SOCS mRNA expressed in different forms of periodontal diseases may determine the stable or progressive nature of the lesions.

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Periodontal diseases are the most common human infectious diseases and are caused by an unbalanced host response to periodontopathogens. Host responses are initiated by the recognition of microbial structures, such as lipopolysaccharide from gram-negative bacteria, which interacts with Toll-like receptors. These trigger intracellular signaling pathways that culminate in an inflammatory cytokine response (1).

One of the cytokines involved in the pathogenesis of periodontal disease is tumor necrosis factor-α (TNF-α), which is present at high levels in both gingival crevicular fluid and periodontal tissues of diseased sites. Studies in rats and primates clearly demonstrate that TNF-α plays a central role in the inflammatory reaction, in the alveolar bone resorption and in the loss of connective tissue attachment in experimental periodontal diseases (2). Several more cytokines are also believed to be involved in the immunopathogenesis of periodontal diseases. While inflammatory (interleukin-1 and -6) and T helper 1 type [interferon-γ (IFN-γ)] cytokines have been associated with higher periodontal disease severity, the opposite effect seems to be exerted by the T helper 2 type cytokines, such as interleukin-4 and -10 (3,4).

The current paradigm states that cytokine balance is determined by the relative levels of pro- and anti-inflammatory cytokines. However, because cytokines are secreted proteins and their binding to specific receptors transduces a signal that mediates their function, stringent mechanisms of cytokine-signaling control are essential for ensuring an appropriate response. The control of cell signaling can be exerted by the suppressors of cytokine signaling (SOCS), which act to attenuate signal transduction as part of a negative feedback loop to inhibit the response to subsequent stimuli (5). The SOCS family consists of eight proteins (SOCS-1 to -7, and cytokine-inducible SH2-domain-containing protein) that are produced in response to a diverse range of cytokines and growth factors. SOCS are rapidly induced on cytokine stimulation and then hinder further signaling by interfering in the janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway, which is critical in signal transduction (5–7).

Individual SOCS are capable of inhibiting multiple cytokines, and it is not clear how the specificity of inhibition is regulated (6,7). SOCS-1 can be induced by multiple cytokines that use the JAK-STAT signaling pathway, including TNF-α, interleukin-6 and IFN-γ, and it has been shown to inhibit the up-regulation of these same inflammatory cytokines (5,8,9). SOCS-3 is strongly induced by interleukin-1, -6, -10 and IFN-γ, and its main role seems to be the attenuation of inflammatory cytokine signaling (5,7,10). Recently, SOCS-2 was found to mediate the anti-inflammatory properties of aspirin-induced lipoxin *in vivo* (11,12). It has also emerged that SOCS gene expression can be induced by a range of other stimuli, including lipopolysaccharide and CpG oligonucleotides (DNA sequences that include unmethylated cytosine-guanosine sequences), and that SOCS-1 is a negative regulator of TLR-4 signaling (6,10,12,13).

Therefore, as SOCS are potentially involved in the control of lipopolysaccharide and cytokine signaling pathways in the periodontium, the present study was designed to investigate the expression of SOCS-1, -2 and -3 in diseased periodontal tissues. With this aim, control subjects, and patients with chronic gingivitis and chronic periodontitis, were selected from subjects scheduled for treatment at the University of Ribeirão Preto Dental School, using standard inclusion and exclusion criteria, as previously described (14,15). The study protocol was approved by the ethics committee of the University of Ribeirão Preto Dental School. Patients with chronic gingivitis ($n = 20$; 12 men and eight women; mean age 56.6 ± 5.52 yr) were characterized by poor plaque control/oral hygiene, high scores of bleeding on probing sites (> 70%), absent or minimal (at most one tooth per sextant attachment loss = 1 mm) bone loss, and no history of tooth loss resulting from periodontitis. Biopsies of gingival tissue of patients with chronic gingivitis were obtained during surgical procedures as a result of prosthetic reasons. Patients with chronic periodontitis ($n = 20$; 11 men and nine women; mean age 49.8 ± 8.19 yr) presented moderate to advanced periodontal disease (at least one tooth per sextant with a probing depth of > 6 mm and attachment loss > 3 mm, and radiographic evidence of extensive bone loss). All patients received basic periodontal therapy, which consisted of oral hygiene instruction, scaling and root planing. Biopsies of gingival tissue of patients with chronic periodontitis were obtained during surgical therapy of the sites that exhibited no improvement in clinical condition and persistent bleeding on probing 4 wk after the basic periodontal therapy. The control group ($n = 16$, seven men and nine women; mean age 24.7 ± 6.16 yr) comprised subjects presenting clinically healthy periodontium, whose biopsies of gingival tissue were taken during surgical procedures for wisdom tooth removal (none of the sampled sites showed bleeding on probing and all showed probing depths of < 3 mm).

After tissue sample collection, total RNA from gingival tissue biopsies was extracted using the TRIZOL reagent (Life Technologies, Grand Island, NY, USA), and complementary DNA was synthesized using 3 μg of RNA in a reverse transcription reaction (Superscript II; Gibco Life Technologies), as previously described (15). Real-time polymerase chain reaction (PCR) quantitative mRNA analyses were performed in an ABI-Prism7000 Sequence Detection System using the SYBR-green fluorescence quantification system (Applied Biosystems, Warrington, UK), and the calculations for determining the relative level of gene expression by reference to β-actin were performed by the cycle threshold method, as previously described (15). The standard PCR conditions were 95°C for 10 min, 40 cycles of 94°C for 1 min, 56°C as the temperature of annealing (tA) for 1 min, and 72°C for 2 min, followed by the standard denaturation curve. The sequences of the primers were designed based on nucleotide sequences in the GenBank database and were used were as follows: SOCS-1, sense CCCTGGTTGTTGTAGCAGCTT, antisense CAACCCCTGGTTTGTGCAA (190 bp, 60°C tA); SOCS-2, sense GGCTGAAGTCGCGTTTTATCA, antisense ATTGCAG-TGAGGCCTGTGTCA (150 bp, 62°C tA); SOCS-3, sense TAAGTATTGGCCAGTCAGGCG, antisense TTCCATCGCTGCTACATTCCT (201 bp, 60°C tA); interleukin-10, sense AGATCT-CCGAGATGCCTTCA,

antisense CCGTGGAGCAGGTGA-AGAAT (307 bp, 58°C tA); TNF-α, sense AAGCCTGTAGCCCATGTTGT, antisense CAGATAGATGGGCTCA-TACC (330 bp, 56°C tA); β-actin, sense ATGTTTGAGACCTTCAACA, antisense CACGTCAGACT-TCATGA-TGG (495 bp, 56°C tA). Results are depicted as the mean mRNA expression from triplicate measurements normalized by internal control β-actin, analyzed using analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test, performed with the GRAPHPAD Prism 4.0 software (GraphPad Software Inc., San Diego, CA, USA). Values of $p < 0.05$ were considered statistically significant.

Our results demonstrate that the expression of SOCS-1, -2 and -3 mRNA was significantly more prevalent and more intense in diseased tissues than in healthy controls (Fig. 1). Data from the literature demonstrate that SOCS expression can be induced by lipopolysaccharide, cytokine and lipoxin signaling (7,11,13,16). In fact, the quantitative and qualitative changes in periodontal biofilm from healthy to diseased conditions are well known and result in intense antigenic challenge in the gingival crevice (17), which could induce SOCS expression. Furthermore, increased levels of cytokines and lipoxins are found in diseased periodontal tissues (4,18). In agreement, both the prevalence and intensity of cytokine mRNA expression were found to be augmented in diseased vs. healthy tissues (Fig. 1).

Based on data from the literature, we suggest that SOCS may act in order to attenuate multiple signaling pathways in diseased periodontium. SOCS interfere with lipopolysaccharide signaling, which is responsible for initiating the inflammatory cascade that culminates in periodontal disease (1,16,19,20). SOCS also mediate direct inhibition of the signaling of inflammatory cytokines (21,22). Previous studies have demonstrated that SOCS-1 deficiency results in severe synovial inflammation and joint destruction and further lethal inflammatory syndrome as a consequence of unchecked IFN-γ signaling (23–25). SOCS-3 has also been implicated in the modulation of arthritis pathogenesis, being able to suppress the induction and development of joint inflammation in mouse models (25,26). Finally, SOCS are involved in the function of outstanding host anti-inflammatory factors. SOCS-3 was described to mediate the inhibitory effects of interleukin-10 on macrophage activation and production of TNF-α and nitric oxide (27,28). In addition, it was recently demonstrated that SOCS-2 is a crucial intracellular mediator of the anti-inflammatory actions of lipoxins (11), and it was also hypothesized that the induction of SOCS-2 represents a general anti-inflammatory pathway responsible for controlling several innate responses. Interestingly, both interleukin-10 and lipoxins are believed to be important anti-inflammatory mediators involved in the attenuation of periodontal disease severity (14,18,29,30).

These pleiotropic effects of SOCS in the regulation of multiple signaling pathways suggest that these proteins could be involved in determining periodontal disease severity. In agreement, the levels of SOCS-1 and SOCS-3 mRNA were significantly higher in tissues from patients with CG than in

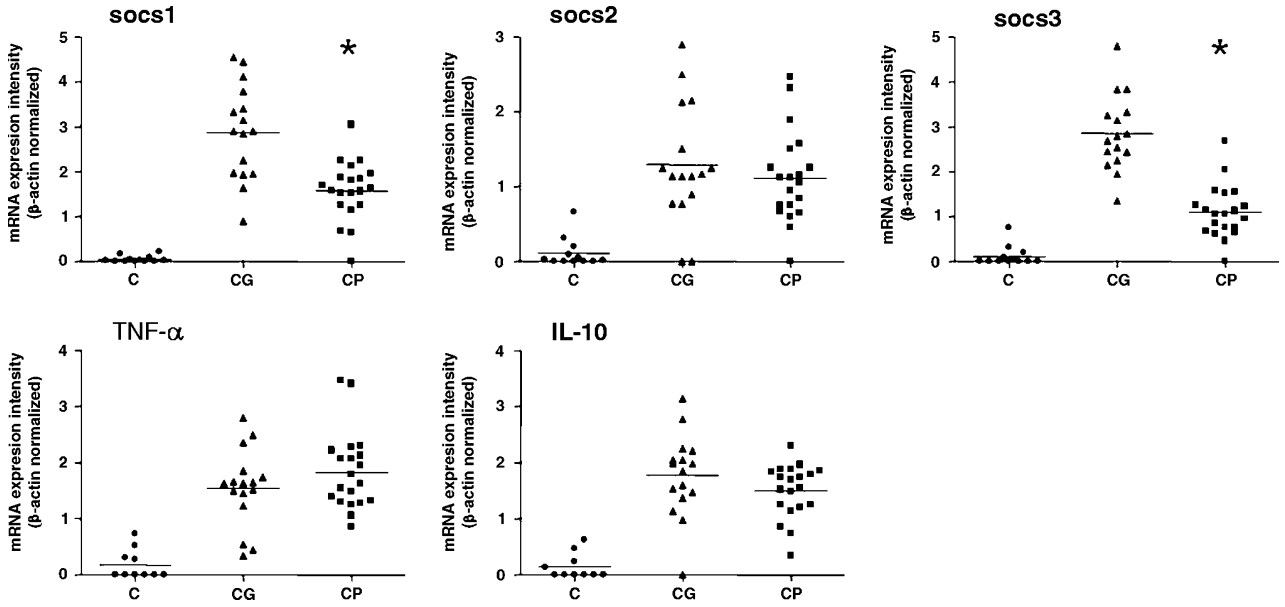

Fig.1. Quantitative expression of suppressors of cytokine signaling (SOCS)-1, -2 and -3, tumor necrosis factor-α (TNF-α) and interleukin-10 in patients with chronic gingivitis (CG) and chronic periodontitis (CP), and in a control group (C). Total RNA was extracted, and the levels of SOCS and cytokine mRNA were measured quantitatively by the RealTimePCR SYBR-Green System. The results are presented as the intensity of expression of the individual mRNAs, with normalization to β-actin, using the cycle threshold method. The results shown are from one experiment representative of three. Controls were significantly different from the CG and CP patients in all analyses. *$p < 0.05$ (CG vs. CP patients).

tissues from those with CP. Furthermore, we also observed negative correlations between the levels of SOCS-1 and SOCS-3 mRNA with clinical parameters of disease severity (probing depth and attachment loss), analyzed through linear regression (data not shown). Gingivitis and periodontitis are considered to be a continuum of the same inflammatory disease (31), but the exact mechanisms responsible for disease progression are not known (32,33). Nevertheless, it has been noted that not all patients with gingivitis will go on to develop periodontitis (31). We believe that the chronic gingivitis group studied, comprising middle-aged to older adult patients who present extensive bleeding on probing but not alveolar bone loss, is representative of some of these periodontitis-resistant subjects. Interestingly, our data demonstrate that the levels of TNF-α and interleukin-10 mRNA were similar between the diseased groups. In spite of some conflicting data, studies demonstrate that TNF-α can be generated by microbial stimulus trough pathways, which are not under the control of SOCS proteins. Therefore, in a scenario with similar levels of pro- and anti-inflammatory cytokines (i.e. gingivitis vs. periodontitis), we suggest that the increased levels of SOCS-1 and -3 mRNA could provide a more effective control of inflammatory signalling in gingivitis than in periodontitis lesions and hence contribute to the nonprogressive nature of gingivitis. Some studies also suggest that changes in the nature of infiltrating lymphocytes and viral infections are involved in the gingivitis–periodontitis transition (32,33). Interestingly, recent studies demonstrate that viral infections can modulate SOCS expression (34,35), which could also contribute to the conversion of a gingivitis site into a tissue-destroying periodontitis lesion. Furthermore, SOCS can also hinder chemokine system signaling (36,37), which could interfere with cell migration patterns and therefore with the local host response.

The present study is the first demonstration of SOCS-1, -2 and -3 mRNA expression in diseased periodontal tissues. Moreover, our results demonstrate a differential expression of mRNA codifying these proteins in different clinical forms of periodontal disease, suggesting their possible involvement in determining the stable or progressive nature of the lesions. However, further studies are now required to confirm such a hypothesis. Indeed, the pro- and anti-inflammatory networks implicated in the immunopathogenesis of periodontal disease are very complex and involve several mediators other than TNF-α, interleukin-10 and SOCS. Besides proteins of the SOCS family, other regulatory molecules, such as sMyD88, IRAK-M, Tollip and TRAILR, have been described as significant negative regulators of inflammatory signaling and could also play important roles in the periodontal disease process. Therefore, knowledge regarding the role of regulators of cell signaling to the outcome of periodontal diseases may provide the basis for future therapeutic interventions aimed at limiting the inflammatory process in periodontal tissues.

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