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The potential role of suppressors of cytokine signaling (SOCS) in the attenuation of inflammatory reaction and alveolar bone loss associated with apical periodontitis

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Abstract

Inflammatory cytokines contribute to periapical tissue destruction. Their activity is potentially regulated by SOCS (suppressors of cytokine signaling), which downregulate signal transduction as part of an inhibitory feedback loop. We investigated the expression of the cytokines TNF- α , IL-10 and RANKL, and SOCS-1, -2 and -3 by Real Time-PCR in 57 periapical granulomas and 38 healthy periapical tissues. Periapical granulomas exhibited significant higher SOCS-1, -2 and -3, TNF- α , IL-10 and RANKL mRNA levels when compared to healthy controls. Significant positive correlations were found between SOCS1 and IL-10, and between SOCS3 and IL-10. Significant inverse correlations were observed between SOCS1 and TNF- α , SOCS1 and RANKL, and SOCS3 and TNF- α . Increased SOCS-1, -2 and -3 mRNA levels in periapical granulomas may be related to the downregulation of inflammatory cytokines in these lesions; therefore, SOCS molecules may have a role in the dynamics of periapical granulomas development.

Keywords

suppressors of cytokine signaling; SOCS; cytokines; TNF-alpha; RANKL; inflammation; periapical granulomas

1. INTRODUCTION

Cytokines play a major role in inflammatory and immune responses within the bone microenvironment. The balance between pro- and anti-inflammatory mediators determines the

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outcome of resorption in bone destructive diseases, including periapical granulomas (1). The recognition of microbial structures such as lipopolysaccharides from Gram-negative bacteria through Toll-like receptors (TLRs) triggers intracellular signaling pathways that culminate in an inflammatory cytokine response and bone lesions (2). Previous studies have demonstrated that proinflammatory cytokines such as receptor activator of NF- κ B ligand (RANKL) and tumor necrosis factor alpha (TNF- α), members of the TNF super family, are established agents in the pathogenesis of chronic inflammatory diseases including pulpitis and apical periodontitis (3–5). TNF- α is a potent immunologic mediator of acute and chronic inflammatory responses (6) and has the ability to increase bone resorption (7). On the other hand, IL-10 is a pleiotropic cytokine with strong anti-inflammatory properties, regulating B-cell proliferation and differentiation and exhibiting immunoregulatory activities (8). IL-10 directly affects osteoclast precursors and inhibits osteoclast generation and activation (9) and has been suggested as an important suppressor factor for periodontal disease (10) and apical periodontitis (11) development in mice.

Activation of cytokine signaling via the Janus kinase (JAK) and activators of transcription (STAT) pathways may represent a key mechanism by which inflammatory cytokines contribute to osteoclast formation. Members of the suppressors of cytokine signaling (SOCS) family, which comprise eight proteins (SOCS-1 to -7, and cytokine-inducible SH2-domain-containing protein-CIS), may inhibit these pathways. The SOCS genes are activated in response to a diverse range of microbial and immunological factors (12–14). Individual SOCS are capable of inhibiting multiple cytokines, although mechanisms of binding affinity are still unclear (13,14). SOCS-1 can be induced by cytokines that use the JAK-STAT signaling pathway, including TNF- α , IL-6, and IFN- γ , and it has been shown to inhibit the upregulation of these same inflammatory cytokines (12,15,16). SOCS-2 mediates the anti-inflammatory properties of aspirin-induced lipoxin *in vivo* (17). SOCS-3 is strongly induced by IL-1, IL-6, IL-10 and IFN- γ , and its main role appears to be the attenuation of inflammatory cytokine signaling (12,14). Some investigators have suggested that SOCS gene expression can be induced by a range of microbial stimuli, including lipopolysaccharides and CpG oligonucleotides, and that particularly SOCS-1 is a negative regulator of the lipopolysaccharide-induced inflammatory effects via inhibition of TLR4 signaling (13,17–19) (Figure 1). While the expression of SOCS in healthy tissues is generally absent or minimal, different degrees of upregulated SOCS expression in inflamed tissues may determine the outcome of inflammatory reaction. Therefore, we hypothesize that a differential expression of SOCS in periapical environment could interfere in the expression inflammatory cytokines such as TNF- α and RANKL, which would be relevant to periapical granulomas outcome.

Therefore, in this study we investigated the expression of cytokine suppressors SOCS-1, -2 and -3 in human periapical granulomas, and their correlations with immunoregulatory cytokines, in order to improve understanding of some of the underlying mechanisms during periapical lesions development.

2. MATERIAL AND METHODS

This study was approved by the Institutional Review Board at Bauru Dental School. Fifty-seven periapical granulomas were collected from previously selected patients aged 15–58 years (average age 23.15 years, 27 females and 30 males) submitted to periapical surgery and curettage of the tissues as part of their clinical treatment. The diagnosis of periapical lesion was based on a radiographic image showing clear bone loss and disappearance of the periodontal ligament space in the periapical region. The collected lesions were divided in 2 parts for histopathological and molecular analyses. Routine histopathologic examination (hematoxylin-eosin staining of serially sectioned specimen) was performed, and granulomas represented a severe infiltration of inflammatory cells with no evidences of epithelial cells, i.e.

epithelial lining and/or epithelial infiltration within the lesion. Periapical cysts were classified as fully developed cavities lined by stratified squamous epithelium and a fibrous capsule, and were excluded from the sample. Partially epithelized lesions, considered in some studies epithelized granulomas, were also excluded, and will be properly analyzed comparatively with cysts and granulomas in a further study. Lesion diameter was obtained by direct measurement using an endodontic ruler onto the periapical radiograph (taken with an intraoral film positioner).

Periodontal ligaments were used as control specimens, obtained from teeth extracted for orthodontic purposes from 38 subjects (20 males and 18 females, age 17–23 years) presenting with good oral health. Patients presenting medical history indicating use of systemic modifiers of bone metabolism, antibiotic, anti-inflammatory, hormonal or other assisted drug therapy within 6 months prior to the study, with primary or secondary acute periodontitis, or females who were pregnant or nursing were not included in the study.

Total RNA was extracted from samples using TRIZOL reagent (Life Technologies, Grand Island, NY, US), and complementary DNA (cDNA) was synthesized using 3µg of RNA through a reverse transcription reaction as previously described (20). Real Time-PCR quantitative mRNA analyses were performed in a MiniOpticon thermocycler (BioRad, Hercules, CA, US) using SYBR-green chemistry (Invitrogen, Carlsbad, CA, US), with specific primers previously described (20). Determination of the relative levels of gene expression was performed using the cycle threshold (Ct) method, in reference to beta-actin, as described elsewhere (20). PCR conditions were: 95°C (10min), 40 cycles at 94°C (1min), annealing at 56°C (1min), and 72°C (2min). Results are depicted as the mean mRNA expression from triplicate measurements normalized by internal control beta-actin. Statistical analyses included ANOVA followed by Bonferroni correction, in GraphPad Prism 4.0 (GraphPad Software Inc, San Diego, CA, US). $P \leq 0.05$ was considered statistically significant.

3. RESULTS

SOCS-1, -2 and -3, TNF- α , IL-10 and RANKL mRNA expression was significantly more frequent and intense in diseased tissues than in healthy tissues ($p < 0.001$) (Figure 2). The levels of IL-10 showed a positive correlation with SOCS1 ($r^2 = 0.1302$, $p = 0.0058$) and SOCS3 ($r^2 = 0.1085$, $p = 0.0124$), while the levels of SOCS1 and TNF- α ($r^2 = 0.1857$, $p = 0.0008$), SOCS1 and RANKL ($r^2 = 0.0923$, $p = 0.0116$), and SOCS3 and TNF- α ($r^2 = 0.1857$, $p = 0.0008$) were negatively correlated (Figure 3). A positive correlation was also observed between TNF- α and RANKL, and a trend for negative correlation was found for IL-10 and RANKL (data not shown). SOCS1 ($r^2 = 0.0705$, $p = 0.0359$) and SOCS2 ($r^2 = 0.0892$, $p = 0.0140$) mRNA levels were inversely correlated with lesion size; however, for SOCS3 only a trend toward inverse correlation was verified ($r^2 = 0.0238$, $p = 0.1516$) (Figure 3).

4. DISCUSSION

Proinflammatory cytokines play a fundamental role in periapical bone destruction through the induction of RANKL, a gene directly involved in osteoclast activation (5,21,22). On the other hand, the protective mechanisms against bone resorption are not fully understood. In this study, we used samples of human periapical granulomas and investigated the expression of SOCS molecules (regarded as potentially protective) and cytokines involved in RANKL induction (TNF- α) and inhibition (IL-10) in order to access their possible role in the bone resorption process associated with these endodontic lesions.

Our results showed that human periapical granulomas presented significantly higher levels of SOCS-1, -2 and -3 mRNA when compared to healthy periapical tissues, indicating a possibly

active role for SOCS in the pathogenesis of periapical granulomas. SOCS proteins are negative regulators of the inflammatory signaling triggered by toll-like receptors (TLRs) and pro-inflammatory cytokines, and attenuate signal transduction as part of a negative feedback loop to inhibit the response to subsequent stimuli (12). Therefore, in accordance with our findings, SOCS are usually absent or minimal in healthy tissues, and their upregulated and differential expression in inflamed tissues may determine the outcome of inflammatory reaction (12). Both SOCS-1 and SOCS-3 negatively regulate the innate and adaptive immune mechanisms in inflammatory arthritis (23), and their deficiency results in increased tissue destruction (24, 25). SOCS-2 is a key intracellular mediator of the anti-inflammatory actions of lipoxins and its induction has been suggested to represent a general anti-inflammatory pathway responsible for controlling several innate responses (26).

Furthermore, the expression of SOCS has been shown to tune pro-inflammatory signals (like TLRs and cytokines signaling) to prevent excessive inflammatory damage to the host tissues (14,17,19,27). Both LPS (which is a TLR-ligand) and several cytokines are released in root canal infection, and are involved in the development of periapical lesions (28). Therefore, we speculate that SOCS may contribute to the regulation of inflammatory molecules in the periapical microenvironment. Accordingly, our results demonstrated an inverse correlation between mRNA levels of SOCS-1 and TNF- α , SOCS-1 and RANKL, and SOCS-3 with TNF- α in periapical granulomas. TNF exacerbates bone resorption through the upregulation of RANKL expression (29). On the other hand, we found a positive correlation between SOCS1, SOCS3 and IL-10. The anti-inflammatory effects of IL-10, which involve inhibition of inflammatory mediators of mRNA transcription, may involve the participation of SOCS proteins, mainly SOCS1 and SOCS3 (30–32). Interestingly, IL-10 attenuates experimental periapical lesion progression (11), suggesting that IL-10-induced SOCS may down regulate the inflammatory signaling that leads to TNF- α mRNA transcription, which in turn could down regulate RANKL levels and interfere with lesion progression. Corroborating our findings, a similar pattern of co-expression of SOCS and cytokines was described in periodontal diseases, where increased levels of SOCS-1 and -3 were associated with non progressive gingivitis lesions (33).

SOCS-1 and -3 levels showed a trend toward negative correlation with lesion size. This could be explained in part by the association of SOCS-1 and -3 with lower RANKL expression. We have recently demonstrated that RANKL/OPG ratio is possibly associated with stable or progressive stages of periapical granulomas (34). Surprisingly, SOCS-2, not associated with RANKL expression, presented a significant negative correlation with lesion size. SOCS-2 was described to mediate the ubiquitinylation and proteasome-mediated degradation of TRAF6 (26), a RANKL-induced transcription factor essential to osteoclast development. Therefore, in addition to modulating inflammatory signaling, it is also possible that SOCS proteins influence signaling pathways involved in osteoclast differentiation and activation (27,35).

To our knowledge, this is the first demonstration of a differential expression of SOCS-1, -2 and -3 mRNA expression in healthy and diseased human periapical tissues. These SOCS proteins were found to be differentially associated with molecules involved in the exacerbation or attenuation of bone loss, suggesting their possible involvement in the pathogenesis of the periapical lesions, as follows: 1) the expression of SOCS in inflammatory cells, induced as a negative feedback in response to inflammatory cytokines and microbial signaling and upregulated by IL-10, blocks further pro-inflammatory signaling and consequently dampens the inflammatory reaction (as exemplified in Fig. 1A); 2) the decrease in inflammatory mediators expression, such as TNF- α , resulting thereafter in a lower expression of RANKL, which in turn attenuates the bone resorption associated with periapical granulomas; and 3) SOCS also can directly on osteoclasts precursors through the inhibition of the TRAF6 and NF κ B, molecules with a essential role in the differentiation and activation of osteoclasts (as

exemplified in Fig. 1B). Therefore, we can consider that SOCS can attenuate periapical lesions progression at multiple steps.

Taken together, our data suggests that SOCS may potentially down regulate signaling events involved in osteoclast differentiation and activation, which consequently could interfere in lesion outcome, pointing to a promising role of SOCS as therapeutic targets to clinically interfere in bone resorption process. Indeed, experiments in a mouse model of periapical lesions, in which SOCS will be both induced and inhibited, are underway to confirm such hypothesis. Furthermore, comparative analysis of SOCS expression in different types of periapical lesions, as well the elucidation of its spatial expression within the lesions certainly will contribute to the understanding of periapical lesions pathogenesis. However, it is also important to consider that the pro- and anti-inflammatory networks implicated in the immunopathogenesis of apical periodontitis are very complex. In addition to proteins of the SOCS family, other regulatory molecules such as sMyD88, IRAK-M, Tollip and TRAILR have been suggested as important negative regulators of inflammatory signaling and could play important roles in apical periodontitis. Noteworthy is that understanding the role of regulators of cell signaling in the development of periapical diseases may provide the basis for future therapeutic interventions.

5. CONCLUSION

Our study demonstrates that SOCS-1, -2 and -3 are differentially expressed in healthy and diseased human periapical tissues, and also differentially associated with molecules involved in the exacerbation or attenuation of bone loss. Based on these findings, we suggest that SOCS may potentially downregulate signaling events involved in inflammatory reaction and osteoclast differentiation and activation, and interfere directly in the development and progression of periapical lesions. Therefore, SOCS are promising therapeutic targets to clinically interfere in periapical bone resorption process.

Abbreviations

RANK	Receptor activator of NFκB
RANKL	Receptor activator of NFκB-ligand
OPG	osteoprotegerin
SOCS	suppressors of cytokine signaling
TNF-α	tumor necrosis factor-alpha
IL-10	Interleukin-10
STAT	signal transducers and activators of transcription
TLRs	Toll-like receptors
CIS	

cytokine-Inducible SH2-domain-containing protein

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A SOCS action in inflammatory cells **B** SOCS action in osteoclast precursors

Figure 1. The mechanisms of SOCS (suppressors of cytokine signaling) action in inflammatory cells and osteoclast precursors

A schematic representation of mechanisms of SOCS (suppressors of cytokine signaling) action in inflammatory cells and osteoclast precursors. A) The inflammatory signaling triggered by LPS or cytokines such as TNF- α and IL-1 β (1) may involve several signaling intermediates such as JAK/STAT, TRAF6 (2) and transcription factors such as NF κ B (3), which initiate specific mRNA transcription (mRNA^a, mRNA^b) at the nucleus (4). The mRNA is translated into proteins, which will exert their biological roles after secretion (5). Inflammatory signaling also induces the expression of SOCS genes (mRNA^{SOCS}) (6), which is increased by IL-10 (7). The conversion of SOCS into non-secreted proteins (8) allows their intracellular action, which consists in the inhibition of inflammatory signaling (9), dampening therefore the inflammatory effects of LPS and cytokines. B) The interaction of RANKL with the receptor RANK (1) expressed by osteoclast precursors leads to the intracellular signaling that involves TRAF6 (2) and the transcription factor NF κ B (3), which leads to osteoclast differentiation and activation, and initiate specific mRNA transcription (mRNA^a, mRNA^b) at the nucleus (4). Activated osteoclasts produce enzymes such as Cathepsin K and MMPs (5), involved in bone resorption. The expression of SOCS (mRNA^{SOCS}) (6), whose inducing factors in osteoclast precursors remain to be identified (7), may impair osteoclast differentiation and activation through the inhibition/degradation of TRAF6 and NF κ B (8).

Figure 2. Quantitative expression of SOCS, TNF- α , IL-10 and RANKL in periapical granulomas
Total RNA was extracted from periapical granulomas (G, N=57) and periodontal ligament control samples (C, N=38), and levels of SOCS1, SOCS2, SOCS3, TNF- α , IL-10 and RANKL mRNA were measured quantitatively by RealTimePCR SYBR-Green system. The results are presented as expression of the individual mRNAs, with normalization to housekeeping gene using the Ct method. * Statistically significant difference between C and G ($p < 0.001$).

Figure 3. Correlations between the expression of SOCS and cytokines in periapical granulomas and with the radiographic diameter of the lesions

Total RNA was extracted from periapical granulomas (N=57)(A), of SOCS1, SOCS2, SOCS3, TNF- α , IL-10 and RANKL mRNA were measured quantitatively by RealTimePCR SYBR-Green system. Linear regression analysis was used to test the correlations between the levels of SOCS and cytokines expression, and also between SOCS and the radiographic diameter of the lesions. Values of p and r^2 are identified in the graphs.