Expression Analysis of Wound Healing Genes in Human Periapical Granulomas of Progressive and Stable Nature

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Abstract

Introduction: Wound healing process involves the activation of extracellular matrix components, remodeling enzymes, cellular adhesion molecules, growth factors, cytokines and chemokines genes. However, the molecular patterns underlying the healing process at the periapical environment remain unclear. Here we hypothesized that endodontic infection might result in an imbalance in the expression of wound healing genes involved in the pathogenesis of periapical lesions. Furthermore, we suggest that differential expression of wound healing markers in active and latent granulomas could account for different clinical outcomes for such lesions. **Methods:** Study samples consisted of 93 periapical granulomas collected after endodontic surgeries and 24 healthy periodontal ligament tissues collected from premolars extracted for orthodontic purposes as control samples. Of these, 10 periapical granulomas and 5 healthy periapical tissues were used for expression analysis of 84 wound healing genes by using a pathway-specific real-time polymerase chain reaction array. The remaining 83 granulomas and all 24 control specimens were used to validate the obtained array data by real-time polymerase chain reaction. Observed variations in expression of wound healing genes were analyzed according to the classification of periapical granulomas as active/progressive versus inactive/stable (as determined by receptor activator for nuclear factor kappa B ligand/osteoprotegerin expression ratio). **Results:** We observed a marked increase of 5-fold or greater in *SERPINE1, TIMP1, COL1A1, COL5A1, VTN, CTGF, FGF7, TGFB1, TNF, CXCL11, ITGA4,* and *ITGA5* genes in the periapical granulomas when compared with control samples. *SERPINE1, TIMP1, COL1A1, TGFB1,* and *ITGA4* mRNA expression was significantly higher in inactive compared with active periapical granulomas ($P < .001$), whereas *TNF* and *CXCL11* mRNA expression was higher in active lesions ($P < .001$). **Conclusions:** The identification of novel gene targets that curb the progression status of periapical lesions might contribute to a more accurate diagnosis and lead to treatment modalities more conducive to endodontic success. *(J Endod 2012;38:185–190)*

Key Words

Apical periodontitis, gene expression, wound healing

Wound healing involves interactions between cells and their surrounding microenvironment, which includes the extracellular matrix and soluble mediators. The host response to irritants follows a cascade of events involved in wound healing including vascular and cellular inflammatory events, cellular migration, proliferation and differentiation, angiogenesis and epithelialization, fibroplasia, matrix deposition and remodeling (1). Under the influence of several genes, cells are directed to differentiate or dedifferentiate, proliferate, or remain quiescent and assume the architecture and function of the damaged organ (2, 3). Among the genes activated during healing events, extracellular matrix (ECM) components, remodeling enzymes, cellular adhesion molecules, growth factors, cytokines, and chemokines have been described as important factors in the wound healing cascade (4, 5). Intriguingly, the same mediators (ie, inflammatory cytokines) that promote wound healing can also trigger tissue destruction (4, 6). In this context, the nature, extent, and duration of the host response seem to play a major role in the determination of healing versus destructive process, and a complex signaling network operates in the determination of lesion outcome (4, 6).

It is well-understood that endodontic failure is mainly due to an infected root canal system, which acts as a reservoir for microbial cells, virulence products, and antigens that collectively evoke and maintain apical periodontitis (7). However, although the proper healing of apical tissues after successful root canal therapy has been characterized by clinical, radiographic, and histopathologic methods (8), the molecular mechanisms (inflammatory-immune response and tissue destructive mechanisms) underlying persistent apical periodontitis, whose characteristics fit within a chronic wound description, remain unclear.

The healing process is thought to begin immediately after tissue injury, but multiple systemic and local factors might disturb the natural course of healing, resulting in a chronic, nonhealing lesion (9, 10), as observed for periapical granulomas.

Here we hypothesized that endodontic infection might result in an imbalance in the expression of wound healing genes involved in periapical lesion pathogenesis. We also believe that the differential expression of wound healing markers in active and/or stable granulomas could account for different clinical outcomes for these lesions. The identification of novel targets that curb the progression status of periapical lesions as reported in this study might improve diagnostic procedures and the development of individualized treatment plans and regenerative procedures.

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Materials and Methods

Subjects and Samples

This study was approved by the Institutional Review Board at Bauru Dental School, University of São Paulo, and University of Ribeirão Preto Dental School (UNAERP). Subjects were patients (age, 15–57 years; average, 38.2 years) with periapical lesions, characterized radiographically as rarefaction lesions with the disappearance of the periodontal ligament space and discontinuity of the lamina dura, and were referred to endodontic surgery after conventional root canal treatment had failed. Patients with medical conditions requiring the use of systemic modifiers of bone metabolism or other assisted drug therapy (ie, systemic antibiotics, anti-inflammatory, hormonal therapy) during the last 6 months before initiation of the study, patients with preexisting conditions such as periodontal disease, and pregnant or lactating women were excluded from the study.

Periapical lesion samples were collected integrally in formalin solution or divided into 2 roughly similar fragments and stored in formalin and RNALater (Ambion, Austin, TX) solutions. Samples stored in formalin were submitted to routine histologic processing (formalin-fixed paraffin-embedded tissues) and serially sectioned for posterior histopathologic analyses. Only cases of periapical granulomas, represented by the presence of capillaries, inflammatory cells, fibroblasts, collagen, and macrophages and without the presence of an epithelial lining, were selected for the study. Periapical cysts, in which cavities were further developed and lined by stratified squamous epithelium, were excluded. A total of 93 periapical granulomas were selected for the study. Healthy periodontal ligament tissue samples (n = 24) obtained from premolars extracted for orthodontic purposes (patients aged 17–23 years) were stored in RNALater solution and used as control specimens.

Gene Expression Analysis

Total RNA was extracted from the tissue samples by using the RNeasy FFPE Kit (Qiagen Inc, Valencia, CA) or TRIZOL reagent (Life Technologies, Grand Island, NY), according to the manufacturers' instructions. Next, the RNA pellet was dried under a vacuum and resuspended in 30–50 μL of diethyl pyrocarbonate (DEPC)–treated water. The integrity of RNA samples was checked by analyzing 1 μg of total RNA on 1.2% (w/v) denaturing formaldehyde-agarose gel. After RNA extraction, complementary DNA was synthesized by using 3 μg of RNA through a reverse transcription reaction.

Initially, 10 periapical granulomas were randomly selected for investigation of messenger RNA (mRNA) expression of 84 wound healing genes as contained in the human wound healing PCR array (PAHS-121; SABiosciences, Frederick, MD) for pathway profiling by real-time reverse transcription polymerase chain reaction (RT-PCR). Healthy periodontal ligament tissue samples (n = 5) were used as controls. Details of the array gene list are presented in Table 1. Real-time PCR array data were analyzed by using the RT^2 profiler PCR Array Data Analysis online software (SABiosciences).

Next, 83 periapical granulomas were used for validation of the results obtained with the PCR array. We investigated the expression of the genes found to be significantly modulated in the PCR array by using real-time PCR and SYBR-green chemistry (Invitrogen, Carlsbad, CA) and specific primers (Table 2). Primers were designed by using Primer Express 2.0 software (Applied Biosystems, Foster City, CA). Reaction conditions were 95°C (10 minutes), 40 cycles at 94°C (1 minute), annealing at 56°C (1 minute) and 72°C (2 minutes). The results are depicted as the fold increase change in mRNA expression from triplicate measurements in relation to control samples and normalized by the internal controls glyceraldehyde-3-phosphate dehydrogenase, hypoxanthine-guanine phosphoribosyltransferase, β-actin, and breast cancer resistance protein by using the cycle threshold method (11). Samples were categorized in putative progressive/active and stable/inactive lesions based on the receptor activator for nuclear factor kappa B ligand/osteoprotegerin (RANKL/OPG) expression ratio as previously described (12). In brief, samples expressing predominantly RANKL (RANKL > OPG group, n = 32 of 83, 38.55%) were considered putative progressive lesions, whereas predominant expression of OPG or similar RANKL and OPG expression levels (RANKL $\cong$ OPG or RANKL < OPG, n = 51 of 83, 61.44%) characterized potentially stable lesions. Healthy periodontal ligament tissue samples (n = 24) were used as controls. Statistical analyses included analysis of variance (ANOVA), followed by Bonferroni correction in GraphPad Prism 4.0 (GraphPad Software Inc, San Diego, CA). A P value $\leq$.05 was considered statistically significant.

TABLE 1. Details of Investigated Genes as Contained in the Human Wound Healing PCR Array

Gene function	Genes
ECM and cell adhesion	
ECM components	*COL14A1, COL1A1, COL1A2, COL3A1, COL4A1, COL4A3, COL5A1, COL5A2, COL5A3, VTN*
Remodeling enzymes	*CTSG, CTSK, CTSL2, F13A1, F3 (tissue factor), FGA (fibrinogen), MMP1, MMP2, MMP7,MMP9, PLAT (tPA), PLAU (uPA), PLAUR (uPAR), PLG, SERPINE1 (PAI-1), TIMP1*
Cellular adhesion	*CDH1 (E-cadherin), ITGA1, ITGA2, ITGA3, ITGA4, ITGA5, ITGA6, ITGAV, ITGB1, ITGB3, ITGB5, ITGB6*
Cytoskeleton	*ACTA2 (a-SMA), ACTC1, RAC1, RHOA, TAGLN*
Inflammatory cytokines and chemokines	*CCL2 (MCP-1), CCL7 (MCP-3), CD40LG (TNFSF5), CXCL1, CXCL11 (ITAC/IP-9), CXCL2, CXCL5 (ENA-78/LIX), IFNG, IL10, IL1B, IL2, IL4, IL6*
Growth factors	*ANGPT1, CSF2 (GM-CSF), CSF3 (GCSF), CTGF, EGF, FGF10, FGF2, FGF7, HBEGF (DTR), HGF, IGF1, MIF, PDGFA, TGFA, TGFB1, TNF, VEGFA*
Signal transduction	
TGF-ß	*TGFB1, TGFBR3, STAT3*
WNT	*CTNNB1, WISP1, WNT5A*
Phosphorylation	*MAPK1 (ERK2), MAPK3 (ERK1), PTEN*
Receptors	*EGFR, IL6ST (GP130)*
Other	*PTGS2*

Results

PCR Array

Of the 84 genes represented in the wound healing PCR array panel, there was a marked increase of 5-fold or greater in 12 genes, *SERPINE1, TIMP1, COL1A1, COL5A1, VTN, CTGF, FGF7, TGFB1, TNF, CXCL11, ITGA4,* and *ITGA5*, in the periapical granulomas when compared with controls (Fig. 1).

Real-Time PCR

The results of the real-time PCR corroborate our findings with the PCR array. When analyzing the periapical lesions collectively, we

TABLE 2. Primer Sequences and Reaction Properties

Target	Sense and anti-sense sequences	tA (°C)	tM (°C)	Bp
COL1A1	AATCACCTGCGTACAGAACGG CAGATCACGTCATCGCACAAC	61	84	114
COL5A1	GGCTCCCGAGAGCAACCT CGGGACACTCACGAACGAA	62	85	55
CTGF	TGCACCGCCAAAGATGGT GACTCTCCGCTGCGGTACAC	62	85	62
CXCL11	CACTTCTTTCCCCAAACATCATG ACAAACCAAATGATGCATAAGAATG	55	75	71
FGF7	CAAGAGCACAATGCCCAAAA CCTCAAGCCTTCATGACATTCA	57	78	71
ITGA4	GCGGAAGAGGACCTCAGTATCA GCTTTAGATTGTCCATTTCCTCTTCA	58	78	70
ITGA5	CAGTGCCGAGTTCACCAAGA GCCTTGCCAGAAATAGCTTCCT	60	82	67
SERPINE1	TCAAAACACAACTTCTCAGTGTCATC AGCTTGCAACATACCAGATATTGC	56	76	75
TGB1	CGCGCATCCTAGACCCTTT CTGTGGCAGGTCGGAGAGA	62	85	57
VTN	CCAGAGCTGCTGCACAGACTA ATCCCCGCGAGTCACTTG	62	84	58
COL5A1	GGCTCCCGAGAGCAACCT CGGGACACTCACGAACGAA	62	85	55
TIMP1	ACTGCAGGATGGACTCTTGCA TTTCAGAGCCTTGGAGGAGCT	30	82	206
TNF	AAGCCTGTAGCCCATGTTGT CAGATAGATGGGCTCATACC	56	79	230
β-actin	ATGTTTGAGACCTTCAACA CACGTCAGACTTCATGATGG	56	75	195

bp, base pairs of amplicon size; tA, annealing temperature; tM, melting temperature.

observed significantly higher expression of the same 12 genes *(SERPINE1, TIMP1, COL1A1, COL5A1, VTN, CTGF, FGF7, TGFB1, TNF, CXCL11, ITGA4,* and *ITGA5*) in the periapical granulomas when compared with controls ($P < .001$, unpaired *t* test, data not shown). Furthermore, expression levels were of similar magnitude than those found in the PCR array analysis.

When comparing active and inactive periapical granulomas, we observed that expression of *SERPINE1, TIMP1, COL1A1, TGFB1,* and *ITGA4* was higher in inactive periapical granulomas ($P < .001$), whereas *TNF* and *CXCL11* expression was higher in active lesions ($P < .001$). The levels of *COL5A1, VTN, CTGF, FGF7,* and *ITGA5* mRNA were similar in both active and inactive periapical granulomas (Fig. 2).

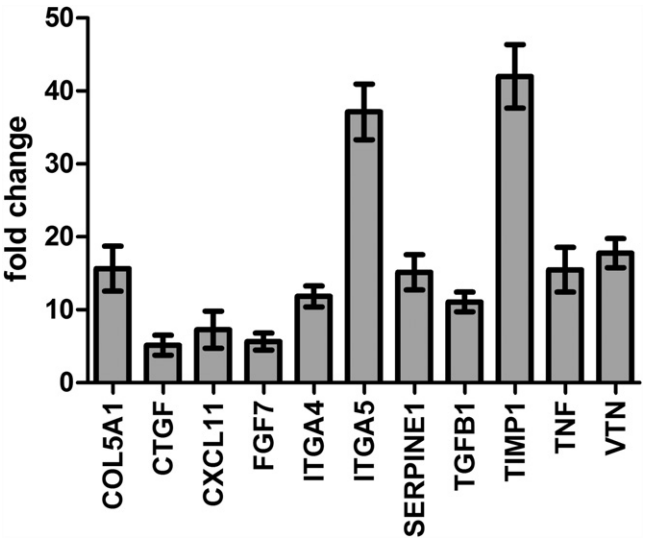

Figure 1. Graphic representation of mRNA expression results obtained by using the human wound healing PCR array. Results are depicted as the fold increase change in mRNA expression from triplicate measurements in relation to control samples and normalized by the internal controls by using the cycle threshold method.

Discussion

Wound healing is a highly ordered and well-coordinated process that involves overlapping phases of inflammation, proliferation, and remodeling, each of which is characterized by dynamic and reciprocal interactions among components of the ECM, growth factors, and cells (13). In this study, we investigated the expression patterns of wound healing genes in periapical granulomas and also the differential expression of those genes in active and inactive lesions. We chose to study periapical granulomas as models of periapical lesions because of the high incidence of granulomas obtained during apical surgery (14).

Our findings corroborate previous reports suggesting that prolonged inflammation might impair the healing of chronic wounds via the adverse action of cytokines affecting growth and viability of cells and impeding the integrity of the ECM (1, 9, 15).

Within a persistent infection scenario, chronic periapical lesions might also experience a reagudization process that is correlated with increased leukocyte infiltration, predominance of neutrophils attracted by a chemokine milieu, as well as increased presence of interleukin-17 (16). Recently, the expression of the midkine (*MK*) gene by inflammatory cells such as macrophages, lymphocytes, and neutrophils has also been reported as an important factor during development of periapical granulomas (17).

The use of high-throughput transcriptomic and proteomic techniques in pulp biology and periapical disease studies has become increasingly popular and will significantly improve understanding of pulp/periapical tissue physiology and pathology (18). By using

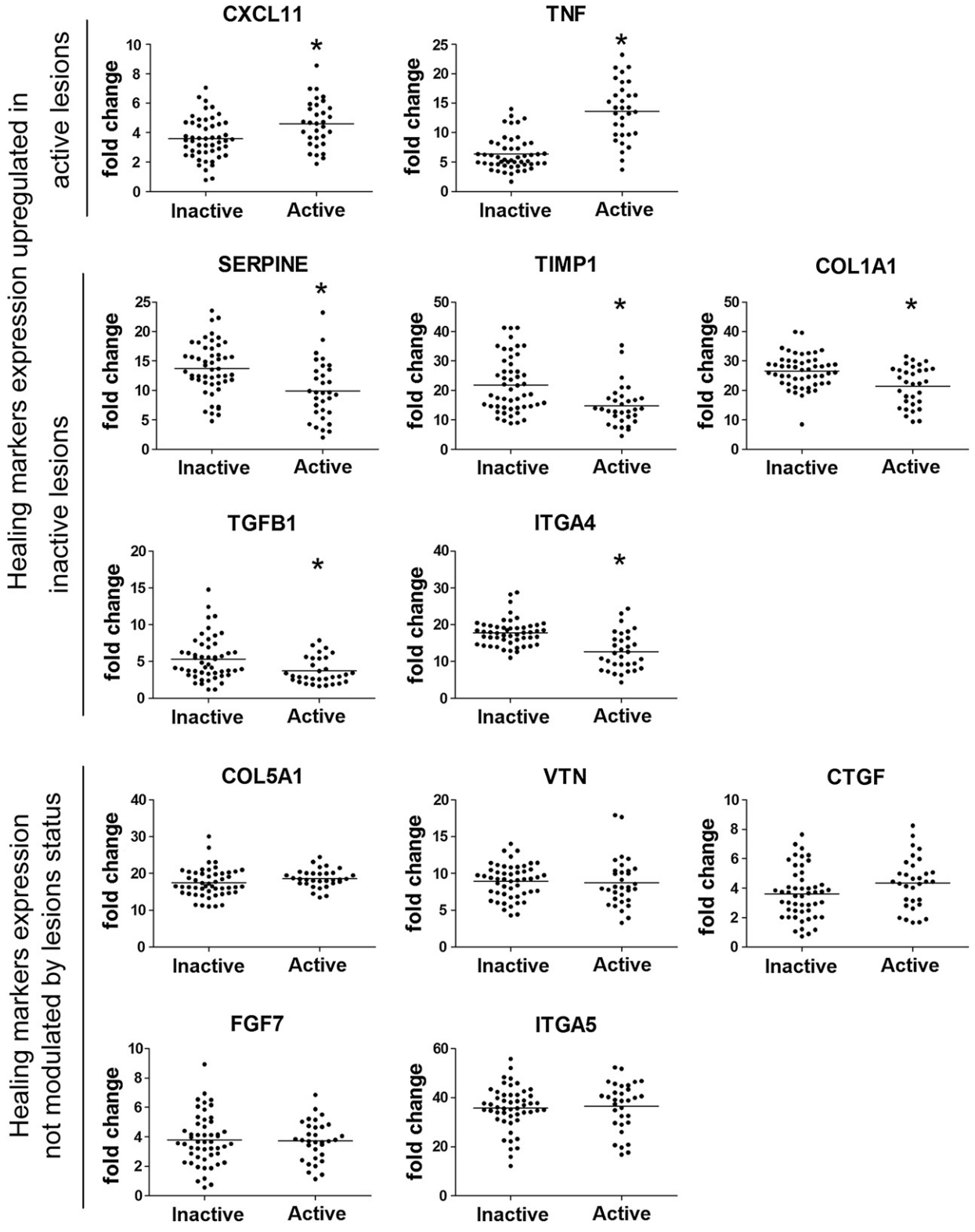

Figure 2. Graphic representation of the expression analysis results by using real-time PCR for the 12 up-regulated genes previously identified in the PCR array in periapical granulomas and healthy periapical tissues (controls). Samples were categorized in putative progressive (active) and stable (inactive) lesions on the basis of the RANKL/OPG expression ratio. Note the marked increase in expression levels of *SERPINE1, TIMP1, COL1A1, TGFB1,* and *ITG4* (*$P < .001$) in inactive lesions and the increased levels of *TNFA* and *CXCL11* in active lesions (*$P < .001$). The remaining genes presented similar expression levels in both active and inactive lesions.

a pathway-specific PCR array technology, we initially explored the expression patterns of 84 wound healing genes in periapical granulomas and healthy periapical tissues, of which 12 genes (*SERPINE1, TIMP1, COL1A1, COL5A1, VTN, CTGF, FGF7, TGFB1, TNF, CXCL11, ITGA4,* and *ITGA5)* were found to be up-regulated in the diseased tissues. These genes have been suggested as important factors in the healing cascade (4, 5) and collectively might represent a healing attempt at the periapical lesion environment.

Chronic wounds are associated with an altered wound milieu resulting from an imbalance in ECM homeostasis. This alteration is characterized by increased destruction and degradation of ECM components, with concomitant lack of synthesis of these elements (19). In this study, *COL1A1*, *COL5A1,* and *VTN* were the most frequently expressed ECM components in the granulomas. *COL1A1* and *COL5A1* are characteristic components of the normal periapical environment and also observed in periapical lesions (20). Collagen V, the protein product of *COL5A1,* has been described to control the initiation of collagen fibril assembly and is therefore an important regulator in the determination of fibril structure and matrix organization (21). The predominant *COL1A1* mRNA expression in inactive lesions could be a reflection of the nonprogressive nature of these lesions. Furthermore, it might represent the closest to what resembles an effective healing process. Vitronectin (*VTN*) is classically associated to cell adhesion and spreading, which can contribute to the healing process by allowing fibroblast transmigration (22) and by interacting with and enhancing the activity of growth factors (23). Still from a cellular adhesion standpoint, our results demonstrate that integrins *ITGA4* and *ITGA5* were significantly overexpressed in the periapical granulomas. Integrins are critical components of the cell attachment machinery, because most normal vertebrate cells cannot survive unless they are anchored to the ECM. Monocyte interactions with the ECM play an important role in inflammatory diseases (24). *ITGA4* was particularly overexpressed in inactive lesions, which suggests that this gene might be important in proper healing for promoting macrophage infiltration, an event that is required during tissue remodeling and considered an important local source of growth factors (24).

The marked increase in the levels of both *SERPINE1* and *TIMP1* in the periapical granulomas is a strong indication of the chronic stage of the investigated lesions (25, 26). *SERPINE1* plays a role in cell adhesion and migration, 2 critical steps to successful wound healing (27). Elevated levels of *SERPINE1* also promote collagen deposition by stimulating the migration of leukocytes and collagen-producing cells into the diseased tissue (28). Interestingly, *SERPINE1* can interact with vitronectin, which was also significantly up-regulated in our sample, and interfere with cellular migration or matrix binding (25).

Delayed healing and formation of chronic wounds have been linked to the excessive production of proteolytic enzymes, leading to reduced amounts of growth factors and successive destruction of the ECM (15, 29). Tissue inhibitor of matrix metalloproteinase 1 (TIMP-1), a member of the TIMP family, has been described as a multifunctional molecule with pleiotropic functions, with roles varying from wound healing and regeneration to a wide range of inflammatory and pathologic processes (26). Matrix metalloproteinases (MMPs) form a multi-gene family within the metalloproteinase class of endopeptidases that collectively mediate the degradation of all ECM molecules (30). TIMP-1 inhibits the proteolytic activity of many MMPs (31). *TIMP1* mRNA was also highly expressed in the periapical granulomas, most often in the inactive lesions. Human TIMP-1 has also been suggested as an enhancer or inhibitor of bone resorption, depending on whether TIMP-1 concentrations are low or high, respectively (32). We believe that the up-regulation of *TIMP1* expression in the periapical granulomas is related to a protective effect against excessive ECM degradation and bone resorption. Despite the potential role of *SERPINE1* and *TIMP1* in either lesion progression or healing attempt, if we consider the higher expression levels of these inhibitors in inactive lesions, it seems reasonable to suggest that both *SERPINE1* and *TIMP1* can play a role, at least in part, in the constriction of progression of periapical granulomas.

Finally, we must consider the action of cytokines and growth factors, possible modulators of the synthesis and remodeling of the ECM, as well as cell migration and proliferation within the wound/lesion site. Collectively, these molecules act as promoters of the wound healing cascade by mediating the selective migration and subsequent activation of leukocyte subsets, endothelial cells, and fibroblasts. Specifically, the expression of *CTGF, FGF7, TGFB1, TNFA,* and *CXCL11* was found to be significantly increased in periapical lesions. *CTGF* and *FGF7* are both potent mitogens that play a role in chondrocyte and keratinocyte proliferation, respectively, and are involved in granulation tissue formation, re-epithelialization, and matrix formation and remodeling (33, 34). Many growth factors such as transforming growth factor beta (TGF-β) and its family members also play a role in tissue repair and regeneration. TGF-β is a potent stimulator for tissue regeneration, with key roles in inflammation, angiogenesis, and re-epithelialization (13). It is also associated with collagen production by inhibiting MMPs and inducing *TIMP1* expression (34). Our results demonstrate that *TGFB1* expression was significantly higher in the inactive lesions. *TGFB1* can be chemotactic and mitogenic for neutrophils, lymphocytes, monocytes, macrophages, and fibroblasts (35, 36). After they have migrated to the wound site, inflammatory cells synthesize and secrete additional *TGFB1*, which in higher concentrations might induce the expression of other growth factors, thereby increasing the cellularity of the wound (37).

Cytokines are also expressed during the healing process (34). In this study, *TNFA* and *CXCL11* were also up-regulated in the periapical granulomas. The gene *TNFA* codes for the proinflammatory cytokine tumor necrosis factor alpha (TNF-α) and is characteristically up-regulated during the inflammatory phase of wound healing. At basal levels, TNF-α can promote wound healing by indirectly stimulating inflammation and increasing macrophage-derived growth factors. At higher levels, however, TNF-α has a detrimental effect on healing because of a predominantly catabolic action, which is mediated by increased production of MMPs that degrade the ECM, thus inhibiting cell migration and collagen deposition. It has been shown that TNF-α and interleukin-1β both stimulate the secretion of MMPs and suppress the production of TIMPs (19). Interestingly, *TNFA* was highly expressed in active periapical granulomas, whereas *TIMP1* was expressed more in inactive lesions. *CXCL11* has been described to be a key regulator of wound repair (38), modulating the synthesis of ECM and basement membrane components, the remodeling and reorganization of collagen matrix, and the re-epithelialization process (34). In dermal healing, *CXCL11* inhibits fibroblast and endothelial cell migration, while promoting migration in keratinocytes (39). However, although these opposing distinct cell responses are required for appropriate dermal healing, such effects might present a detrimental effect in healing of the periapical environment, where fibroblasts and endothelial cells are expected to migrate into the healing tissue. Indeed, the expression of *CXCL11* was significantly higher in active lesions.

Taking into consideration that wound healing is a complex process, we suggest that several genes might contribute to healing, including but not limited to the genes investigated in this study. Finally, the present study provides insights to a variety of genes whose modulation might be therapeutically beneficial to the repair process in the near future.

Conclusion

Wound healing genes play an important role during development of periapical granulomas. The identification of novel gene targets that curb the progression status of periapical lesions might contribute to a more accurate diagnosis and lead to treatment modalities more conducive to endodontic success.

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The authors deny any conflicts of interest related to this study.

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