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Expression of MMP-8 and MMP-13 in the development of periradicular lesions

H. Matsui, M. Yamasaki, K. Nakata, K. Amano & H. Nakamura

Department of Endodontics, School of Dentistry, Aichi-Gakuin University, Chkusa-ku, Nagoya, Japan

Abstract

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Aim To elucidate the expressions of MMP-8 and MMP-13 in experimentally induced rat periradicular lesions by means of the reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemical staining.

Methodology Thirty rats were used and periradicular lesions in mandibular first molar teeth were established following pulp exposure. The animals were sacrificed at 0 (no exposure control), 1, 2, 3, 4 and 6 weeks after pulp exposure. The right molars were used for RT-PCR analysis of MMP-8 and MMP-13. The left molars were subjected to immunohistochemical staining with both MMPs. The areas of these lesions were measured histometrically, and the numbers of both reactive cells in the periapical portion were counted per unit area. Significant differences were analysed by the Mann–Whitney *U*-test.

Results MMP-8 gene expression gradually increased from 2 to 4 weeks, but slightly decreased at 6 weeks. MMP-13 gene expression gradually increased from 1 to 3 weeks. At 4 and 6 weeks, the level of expression was as high as that at 3 weeks. Immunohistochemically, MMP-8 was first detected at 2 weeks and gradually increased until 4 weeks. MMP-13 gradually increased from 1 to 4 weeks. Both MMPs decreased at 6 weeks. The area of the periradicular lesions gradually increased from 1 to 4 weeks, showing a large increase in week 2 and 3 in particular, but then decreased in week 6. MMP-13-expressing cells were significantly greater than MMP-8-positive cells at week 1 and 2.

Conclusions These findings indicate that MMP-8 and MMP-13 were related to the development of periradicular lesions. It is suggested that MMP-13 increased from an early stage during their development and that MMP-8 is involved in the progression of tissue destruction including bone resorption.

Keywords: MMP-13, MMP-8, PCR, periradicular lesion, rat.

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Introduction

Periradicular lesions occur when periapical tissues react to bacterial infection of the dental pulp and root canal, and involve inflammation and alveolar bone destruction. The destruction of periapical tissues might be related to the degradation of the extracellular matrix (ECM), which is the main component of connective tissue (Tjäderhane *et al.* 2007).

Matrix metalloproteinases (MMPs) are thought to play a central role in the breakdown of the ECM (Broverman *et al.* 1998). MMPs consist of five subgroups: collagenases, gelatinases, stromelysins, membrane-type MMPs and other MMPs. MMPs are Zn^{2+}- and Ca^{2+}-requiring enzymes capable of degrading almost all ECM and basement membrane components during normal tissue remodelling and tissue-destructive diseases, including odontogenic tumours and cysts (Wahlgren *et al.* 2001).

Collagenases are the only members of the MMP family that degrade native fibrillar collagens of types I, II and III (Franchimont *et al.* 1997, Johansson *et al.*

Correspondence: Hiroshi Nakamura, Department of Endodontics, School of Dentistry, Aichi-Gakuin University, 2-11 Suemori-dori, Chkusa-ku, Nagoya, 464-8651 Japan (Tel.: +81 52 759 2148; fax: +81 42 764 2299; e-mail: nakaendo@dpc.agu.ac.jp).

1997, Tsubota *et al.* 2002). MMP-1, MMP-8 and MMP-13 comprise the collagenase subfamily in humans, whilst only two of them have been identified in rats and mice (Woessner & Nagase 1994, Jeffrey 1998).

MMP-8 (neutrophil collagenase) was originally believed to be confined to neutrophils (Mainardi *et al.* 1991), but recent studies have indicated that it may be expressed in other cell types, such as osteoarthritic chondrocytes, articular chondrocytes, synovial fibroblasts, endothelial cells and odontoblasts, and dental pulp cells (Hanemaaijer *et al.* 1997, Shlopov *et al.* 1997, Palosaari *et al.* 2000, Wahlgren *et al.* 2002).

MMP-13 was originally discovered in breast cancer (Freije *et al.* 1994). MMP-13 specifically expressed by tumour cells in squamous cell carcinoma of the head and neck and its expression correlates with their invasive capacity. MMP-13 has also been reported in human odontogenic cysts, and its expression has often been related to the biological behaviour of the lesions. It was expressed by plasma cells, fibroblasts, macrophage-like cells, epithelial cells and osteoblasts (Sasano *et al.* 2002, Leonardi *et al.* 2005).

In previous studies, the expression of MMP-13 has been described in human radicular cysts (Leonardi *et al.* 2005). However, little is known about its expression in the temporal development of periradicular lesions. The present study aimed to elucidate the expression of MMP-8 and MMP-13 in experimentally induced rat periradicular lesions by means of the reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemical staining.

Materials and methods

Sample preparation

Thirty male Wistar rats, each weighing approximately 250 g, were used. The rats were anaesthetized with ether, and their mandibular first molar pulps were exposed using a size 1/2 round bur and left open to the oral environment. The animals were humanely sacrificed at 0 (no exposure control), 1, 2, 3, 4 and 6 weeks after pulp exposure (five rats in each period) and their mandibles removed. Their right first molars were used for RT-PCR analyses, and their left ones were subjected to immunohistochemical staining.

Reverse transcription-polymerase chain reaction

The mesial root and surrounding tissues of first molar extracted from the mandible of each rat were combined, and total RNA was isolated with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) before being removed using DNase. A cDNA was constructed from 2 mg of total RNA using Long Range 2 step RT-PCR (Qiagen, Hilden, Germany) and subjected to PCR using Longe Range PCR Enzyme Mix (Qiagen). Specific primers for rat MMP-8 and MMP-13 were designed based on the published DNA sequences: rat MMP-8 sense primer: 5′TCCTTGCCCATGCCTTTCAA3′ and antisense primer 5′CCAAACTATGCTTACAGAGAACCC3′, expected product size: 197 bp; rat MMP-13 sense primer: 5′AGAAGTGTGACCCAGCCCTATC3′ and antisense primer: 5′GCATACGAGCATCCATCCCGA3′, expected product size: 185 bp. The specific primers for rat glyceraldehydes-3 phosphate dehydrogenase (GAPDH) were the same as those described by Tsubota *et al.* (2002): sense primer: 5′CATTCATCCTTGCCTCTCA3′ and anti-rat GAPDH sense primer: 5′GGGCTGTCAG-TCTTGGAAA3′, expected product size: 69 bp. The PCR involved one cycle of denaturation for 3 min at 95 °C, followed by 37 cycles for MMP-8 and 32 cycles for MMP-13 of an amplification sequence that consisted of denaturation for 30 s at 95 °C, annealing for 30 s at 63 °C for MMP-8 and 65 °C for MMP-13 and extension for 1 min with an additional 5 min for the last cycle. The expression level of each gene was normalized to the corresponding GAPDH expression level. Following amplification, equal amounts of PCR products were size separated by electrophoresis through a 2.5% agarose gel and visualized by ethidium bromide staining and ultraviolet transillumination.

Immunohistochemistry

The left mandibles of all animal were fixed with periodate–lysine–paraformaldehyde solution at 4 °C for 24 h. The mandibles were demineralized in 0.5 mol L^{-1} EDTA solution until complete decalcification had occurred. The decalcified tissues were washed in deionized phosphate-buffered saline (PBS) containing 20% sucrose, embedded in O.C.T. compound (Miles Scientific, Naperville, IL, USA) and sectioned serially at 7 μm in the mesiodistal plane. Then, the sections were immunohistochemically stained using an ABC Staining System (sc-2018; Santa Cruz Biotechnologies, Santa Cruz, CA, USA).

The primary polyclonal antibodies used were anti-goat MMP-8 (sc-8848; Santa Cruz Biotechnologies) and anti-goat MMP-13 (sc-31811; Santa Cruz Biotechnologies). Breast cancer sections were used as positive control for each MMP. Normal goat serum was

employed as a negative control. The slides were exposed to hydrogen peroxide in PBS for 10 min to quench the endogenous peroxidase activity and subsequently reacted with 1.5% goat serum diluted in PBS for 1 h to block non-specific antibody binding. These were incubated with each primary antibody 1/100 diluted in PBS with 1.5% goat serum for 12 h, followed by biotinylated antibody for 30 min. Then, they incubated with the avidin-biotinylated peroxidase complex for 30 min. For the final chromogenic reaction, the slides were exposed to freshly prepared substrate solution that consisted of diaminobenzidine-tetrahydrochloride and hydrogen peroxide and then counterstained with haematoxylin.

The periapical tissues of the mesial root of the mandibular first molar were observed histopathologically and immunohistologically examined for both types of antigen-positive cells.

Histometry

Quantitative analysis was performed on five serial sections from each molar in the area of the lesion, namely the periodontal ligament between the root apex and alveolar bone in the mesial root. The area of the periapical lesion was histometrically measured with an image-processing system consisted of a light microscope (BX50F4 Olympus, Tokyo, Japan), digital camera (C-4040 Olympus) and personal computer. The area of these lesions determined with NIH image. The number of cells reacted for antibody of MMP-8 and MMP-13 in the lesions counted by light microscopic observation. An area 0.5 mm-square of periapical portion surrounding the root apex of the mesial root was examined and the cell count per unit area was calculated.

Statistical analysis and animal protocol

The results of all measurements were presented as mean values ± standard deviation. All measurements were made on four serial sections from each animal. The mean value was determined for each animal as well as each group. The statistical significance of differences was determined by the Mann–Whitney *U*-test.

The principles of laboratory animal care according to the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the animal protocol institutionally approved by the Ethics Committee of the School of Dentistry, Aichi-Gakuin University were followed.

Result

Reverse transcription-polymerase chain reaction

MMP-8 gene expression of periapical area was observed at a low level in normal rat and in 1 week after pulp exposure. Its expression of periapical area gradually increased from 2 to 4, but slightly decreased at 6 weeks (Fig. 1).

MMP-13 gene expression was observed at a low level in the normal periapical area. Its expression gradually increased from 1 to 3 weeks after the exposure. At 4

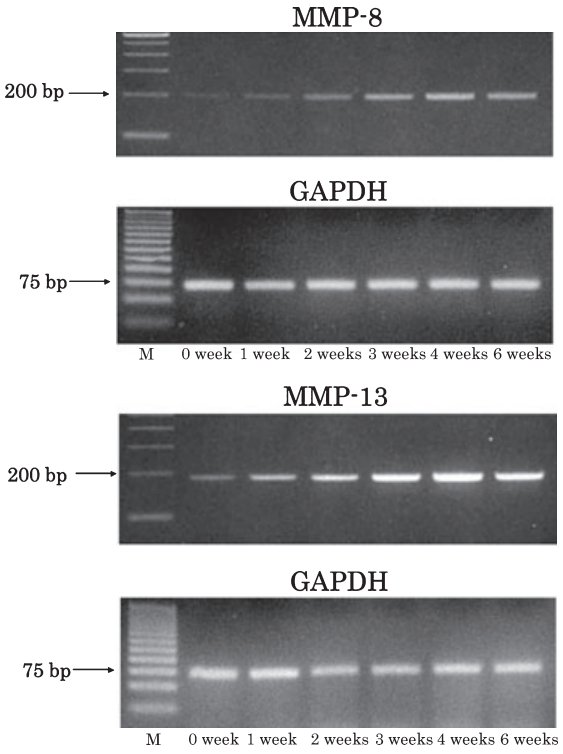

Figure 1 Reverse transcription-polymerase chain reaction analysis of MMP-8 and MMP-13 expression. MMP-8 gene expression is observed in the normal periapical area at a low level in the normal rats. Its expression at 1 week after pulp exposure is as low as normal one. Its expression gradually increases from weeks 2 to 4, but is slightly decreased at 6 weeks. Those expressions after 2 weeks are higher than normal one. MMP-13 gene expression is observed at a low level in the normal periapical area. Its expression gradually increases from 1 to 3 weeks after pulp exposure. At 4 and 6 weeks, the level of MMP-13 expression is as high as that observed at 3 weeks. Those expressions after 1 week are higher than normal one.

and 6 weeks, the level of expression was as high as that at 3 weeks.

Histological findings

At 1 week after pulp exposure, the half of the pulp was necrotized. By 2 weeks, the pulp was almost completely necrotized, and slight inflammation and alveolar bone resorption were observed in the periapical tissue. At 3 weeks, small abscesses were found around the root apex, and severe inflammation and alveolar bone resorption were observed. At 4 weeks, the apical abscess and alveolar bone resorption had extended. By 6 weeks, the abscess and alveolar bone resorption had decreased.

Immunohistochemical findings

MMP-8-expressing cells were not detected in the periapical tissue at 1 week after pulp exposure. At 2 weeks, these cells were presented around the root apex in periapical tissue. By 3 weeks, these cells had increased in number and were observed in and around the apical abscesses. At 4 weeks, these were still observed in and around the abscesses. By 6 weeks, MMP-8-positive cells had decreased, but some still remained in and around the abscesses (Fig. 2).

MMP-13-expressing cells were presented around the alveolar bone of the apical area in the periapical tissue at 1 week after the pulp exposure. At 2 and 3 weeks, these cells had increased in number and were observed in periradicular area. By 4 weeks, these were also seen around the apical abscesses. At week 6, MMP-8 reactive cells had decreased in number, but some still remained around the abscesses (Fig. 3).

Histometrical findings

The area of the periradicular lesion gradually increased from 1 to 4 weeks after pulp exposure, showing a large increase in weeks 2 and 3 in particular, but then decreased in week 6. There were significant differences between the normal and pulp exposure groups after 2 weeks ($P < 0.05$) (Table 1).

At 1 week after pulp exposure, number of MMP-8-expressing cells per unit area in the periapical portion did not increase. From 2 to 4 weeks, their number gradually increased. At 6 weeks, their number decreased. There were significant differences between the normal and pulp exposure groups after 2 weeks ($P < 0.05$).

At 1 week after the pulp exposure, the number of MMP-13-expressing cells per unit area in periapical portion increased. From 1 to 4 weeks, their number gradually increased, but their number decreased at 6 weeks. There were significant differences between the normal and pulp exposure groups ($P < 0.05$).

At 1 and 2 weeks after pulp exposure, there were more MMP-13-expressing cells than MMP-8-expressing cells. There were significant differences between MMP-13 and MMP-8 ($P < 0.05$).

Discussion

In the present study, periradicular lesions were experimentally induced in rat molars, and collagenases (MMP-8 and MMP-13) were detected in the lesions. The results suggest that collagenases participate in tissue destruction during the formation of the lesions.

Experiments using rats have often been conducted to clarify the mechanism of periapical periodontitis: pulp tissues in the molars of rats were exposed, and the exposed areas were left open to induce periapical periodontitis. This reproducible method for inducing periapical periodontitis enables researchers to observe lesions over time and to detect the localization of immunocompetent cells and physiologically active substances immunohistologically. Many studies on the observation of such experimentally induced periradicular lesions have therefore been reported (Yamasaki *et al.* 1994, Kawashima *et al.* 2007).

In this study, the lesion was slightly increased from week 1 to 2 and markedly increased from second to third weeks and from 3 to 4 weeks. The expansion from week 2 to 3 was especially large. The lesion area slightly decreased from week 4 to 6. Based on these results, the period from the start of pulp exposure until week 2 corresponds to the initial phase of inflammation; the period from week 1 to 3 corresponds to the acute phase, in which lesions expand; and the period from 4 to 6 weeks is the chronic phase, in which there is little change in the lesion area.

The level of MMP-8 mRNA expression during the experiment was found to be slightly higher 1 week after the start of pulp exposure, compared with the control group. The level of mRNA expression increased gradually during weeks 2, 3 and 4 after pulp exposure, and the level of expression decreased slightly by week 6. As for the level of MMP-13 mRNA expression, it gradually increased in weeks 1, 2 and 3 after the start of exposure. The level of expression at 4 and 6 weeks after pulp exposure was as high as that at 3 weeks.

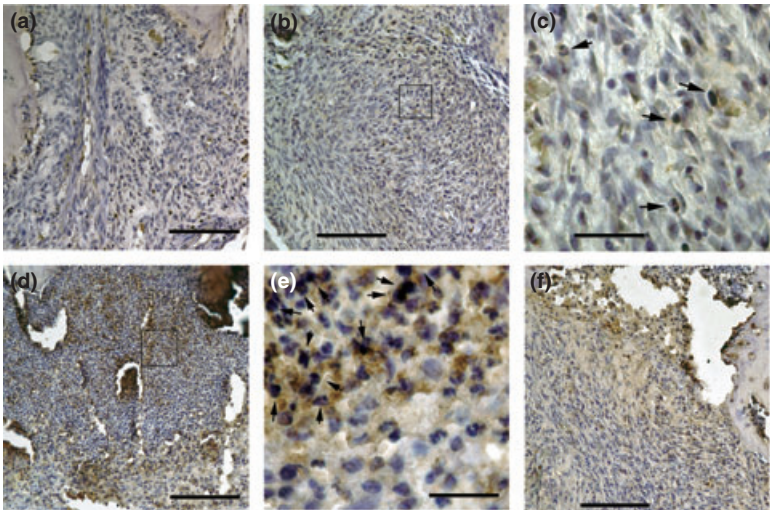

Figure 2 Immunohistochemical staining for MMP-8. In the mandibular first molar at 1 week after the exposure, MMP-8-expressing cells were not detected in the periapical tissue (a). At 2 weeks, MMP-8-positive cells were detected around the root apex (b). (c) is high-magnification view of cropped area in (b). Arrows indicate MMP-8 reactive cells. These cells are mostly polymorphic shaped. At 4 weeks, these increased and were observed in and around the apical abscess (d). (e) is high-magnification view of cropped area in (d). Arrows indicate MMP-8-positive cells that are mostly polymorphic-shaped polynuclear cells. At 6 weeks, these cells still remained in and around the abscesses (f). (a, b, d, and f) scale bars = 0.12 mm. (c and e) scale bars = 0.01 mm.

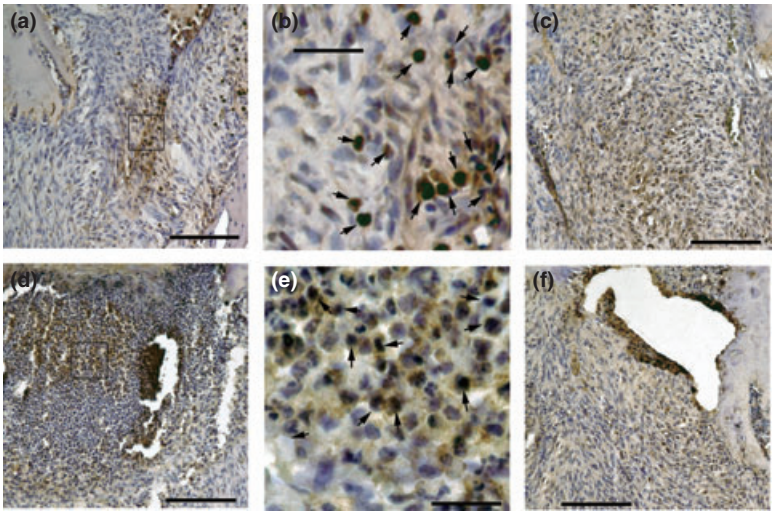

Figure 3 Immunohistochemical staining for MMP-13. At 1 week after the pulp exposure, MMP-13-expressing cells were detected around the alveolar bone in the apical area (a). (b) is high-magnification view of cropped area in (a). Arrows indicate MMP-13-positive cells which are round-shaped monocytes. At 2 weeks, these increased and were detected around the root apex and along alveolar bone (c). At 4 weeks, these cells increased and were observed in and around the apical abscesses (d). (e) is high-magnification view of cropped area in (d). Arrows indicate MMP-13-positive cells. These are almost round-shaped monocytes. At 6 weeks, these cells were present, but some still remained in and around the abscesses (f). (a, c, d and f) scale bars = 0.12 mm. (b and e) scale bars = 0.01 mm.

This suggests that MMP-8 and MMP-13 are involved in the process of periradicular lesion formation. The results also confirmed that the level of MMP-8 mRNA and MMP-13 mRNA expression was higher during the acute phase, in which the periapical lesions expanded, than the initial phase of inflammation.

In this study, MMP-8 and MMP-13 were also investigated immunohistochemically. Few MMP-8-expressing

Table 1 Histometrical results (mean ± SD)

Experimental periods	0 week	1 weeks	2 weeks	3 weeks	4 weeks	6 weeks
Area of periradicular leson (mm^2)	0.19 ± 0.045	0.25 ± 0.121	0.42 ± 0.174	0.81 ± 0.162	1.04 ± 0.193	0.87 ± 0.247
Number of MMP-8 expressing cells (cells mm^{-2})	2.82 ± 1.92	8.31 ± 6.87	27.1 ± 9.64	77.6 ± 23.7	102.5 ± 31.8	84.4 ± 18.6
Number of MMP-13 expressing cells (cells mm^{-2})	3.29 ± 2.07	23.8 ± 10.2	52.4 ± 16.8	79.1 ± 27.5	121.4 ± 32.9	92.4 ± 32.7

cells were observed in periapical tissues 1 and 2 weeks after the start of pulp exposure. Three and 4 weeks after pulp exposure, there was an obvious increase in the number of MMP-8-expressing cells in the periapical tissue. At 6 weeks, however, there was a slight decrease in the number of MMP-8-expressing cells compared with that at 4 weeks. The area of the periradicular lesion gradually increased from 1 to 4 weeks, showing a large increase in weeks 2 and 3 in particular, but then decreased in week 6. These findings suggested that MMP-8 was related the tissue destruction including bone resorption. MMP-8 expression may be involved in the progression of tissue destruction. The pulp exposure induces bacterial infection into pulp tissue, resulting in the infiltration of neutrophil into the periapical tissue and the formation of abscesses at 3 weeks after the exposure. Activated neutrophils assembled in the apical foramen and phagocytized the bacteria present (Yamasaki *et al.* 2008). As a result of their degranulation, such cells might secrete a higher amount of MMP-8.

Neutrophils migrate into tissue through the blood vessels. Neutrophils are phagocytes capable of ingesting microorganisms and particles. In the process of the digestion of microorganisms or particles, specific granules and then azurophil granules fuse with phagosomes, releasing their contents. There have been several reports on neutrophil-derived proteinases in relation to periodontal disease, apical periodontitis and chronic articular rheumatism (Velvart & Fehr 1987, Cootauco *et al.* 1993, Soder 1999). Rauschenberger *et al.* (1994) reported that they found higher levels of neutrophil elastase and cathepsin G expression in human inflamed pulp than in healthy tissue.

However, there were few MMP-13-expressing cells in the area adjacent to the alveolar bone 1 week after pulp exposure. The number of MMP-13-expressing cells increased 2 weeks after pulp exposure. At 3 and 4 weeks, the number of MMP-13-expressing cells further increased in apical abscesses and their peripheries. At 6 weeks, there remained a high number of MMP-13-expressing cells, but slightly fewer than that at 4 weeks.

The process of periapical lesion formation in rat molars can be divided into the initial, acute and chronic phases (Morimoto *et al.* 2008). In previous reports, during the initial phase of rat periapical lesions, inflammatory cells, including macrophages, were detected in the periapical tissue, and the accumulation of macrophages reached its highest level prior to pulp necrosis (Suzuki *et al.* 1999). In this study, the expression of MMP-13 was observed 1 week after the start of pulp exposure and its expression increased 2 weeks after pulp exposure. These results suggest that the resident macrophages in the periapical tissue are activated and differentiated during the initial phase, leading to the secretion of MMP-13 by macrophages and other inflammatory cells. It has been reported that, in radicular cysts, MMP-13 was expressed in the epithelium as well as in plasma cells and fibroblasts in lesions, suggesting that MMP-13 expression is involved in the formation of periapical lesions and that it is also involved in the conversion of periapical lesions into radicular cysts (Leonardi *et al.* 2005). In human odontogenic cysts, the level of MMP-13 expression in plasma cells was higher than that of MMP-8, and mRNA expression of MMP-13 was also stronger than that of MMP-8 in the inflammatory cytokines, IL-6 and TNF-alpha which were produced by plasma cells *in vivo*, according to previous reports (Wahlgren *et al.* 2001).

Based on the results of this study, these confirmed that both MMP-8 and MMP-13 exist in rat periapical lesions. The expression levels of both MMP-8 and MMP-13 increased as the lesion area expanded. MMP-13 expression was observed at an earlier stage than MMP-8 expression. This suggests that MMP-13 initiated the development of periradicular lesions and that MMP-8 was related to this expansion. The present study indicates that MMP-8 and MMP-13 were related to the development of periradicular lesions.

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