Immunohistochemical expression of matrix metalloproteinase MMP-2 and MMP-9 in healthy and inflamed human dental pulp

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Abstract

Objective: The aim of this study was to investigate the impact of inflammation on expression of MMP-2 and MMP-9, as well as to identify the cellular sources of these enzymes in human dental pulps using immunohistochemistry.

Methods: Fifty-four irreversibly inflamed samples of dental pulp were used as the experimental group. Fifty-one healthy pulps, obtained from teeth extracted for orthodontic reasons, were used as the control group. The tissue samples were formalin-fixed, paraffin-embedded, and cut into sections at 3–4 μm. An immunohistochemical study was performed using monoclonal antibodies against MMP-2 and MMP-9. Evaluation of the immunohistochemical expression was determined by the semi-quantitative method and scored as follows: no staining (score 0), less than 10% of stained cells (score 1), less than 30% of weakly stained or strongly but incompletely stained cells (score 2), and more than 30% of strongly and completely stained cells (score 3).

Results: Immunohistochemical analysis revealed a significantly greater expression of MMP-9 in inflamed than in healthy dental pulps (Mann–Whitney U, \( p = 0.0001 \)). In contrast, there was no difference in the expression of MMP-2 between these two groups (Mann–Whitney U, \( p = 0.907 \)). MMP-2 and MMP-9 immunoreactivity was detected the most frequently in endothelial cells.

Conclusions: MMP-9 is highly overexpressed in inflamed dental pulps. There are no differences in the expression of MMP-2 between healthy and inflamed dental pulps. Endothelial cells represent the major cellular source of MMP-9, as well as MMP-2, in healthy and inflamed dental pulps.

Keywords: dental pulp, inflammation, MMP-2, MMP-9, immunohistochemistry

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Introduction

Matrix metalloproteinases (MMPs), collectively known as matrixins, are a family of structurally related enzymes dependent on zinc, which are capable of disintegrating different components of the extracellular matrix (ECM). These enzymes are involved in both normal and pathological tissue remodeling [1].

The fundamental role of MMPs during the development and remodeling of oral tissues has been demonstrated in several studies. These enzymes are involved in the development of enamel and enamel fluorosis [2,3]. In addition to that, MMPs also participate in the remodeling of the organic dentin matrix. It has been proven that activation of MMP-2 and MMP-9 plays a key role in the degradation of dentin collagen during caries progression [4]. The expression of MMPs strongly correlates to periodontal diseases, since they are a major factor in the breakdown of collagen during periodontal tissue destruction [5,6]. Controlled remodeling of the ECM, which is essential for the growth and invasion of oral tissue tumors, is mediated through the activity of MMPs [7]. Evidence of collagenolytic and gelatinolytic activities in partially demineralized dentin, treated with either etch-and-rinse or self-etch adhesives, confirms the participation of these endoproteases in the disruption of incompletely resin-infiltrated collagen fibrils within the hybrid layer [8,9].

The inflammatory process in the dental pulp leads to degradation of the ECM proteins. Different endopeptidases, which act on the various structural proteins, are included in this matrix turnover. Thus, the gelatinases (MMP-2 and MMP-9) are involved in the degradation of denatured gelatins: laminin, elastin, fibronectin and basement membrane zone-associated collagen. Previous studies have suggested that MMPs may play an im-
portant role in pulpitis development and progression [10-14].

The aim of this study was to investigate the impact of inflammation on the expression of MMP-2 and MMP-9, as well as to identify the cellular sources of these enzymes in human dental pulps, using monoclonal mouse anti-human antibodies.

**Material and Methods**

**Sample selection**

The study was approved by a local ethics committee (School of Dentistry, Sarajevo, Bosnia and Herzegovina, number of approval letter: 09-622-2/11) and performed in accordance with the guidelines of the Declaration of Helsinki for Human Research.

All samples were collected prospectively during regular therapy of patients who visited the School of Dental Medicine in Sarajevo for a period of one calendar year. The samples of inflamed pulp were collected during endodontic treatment. The samples of healthy pulp were obtained from caries-free premolars after extraction. Histological and immunohistochemical analysis were performed at Department of Pathology at the Faculty of Medicine in Sarajevo.

Fifty-four (54) samples of dental pulps from premolars, clinically diagnosed as irreversibly inflamed, were used as the experimental group. The diagnosis criteria included: history of spontaneous and/or lingering pain in response to cold and/or heat stimulus, clinically and radiographically evident carious exposure of the pulp chamber, and radiographically normal periapical appearance. Fifty-one (51) samples of healthy pulp extracted from clinically healthy premolars with fully developed roots, which were extracted for orthodontic reasons, were used as the control group.

Immediately after the extirpation, the tissue was placed in 10% neutral buffered formalin, embedded in paraffin and cut with a microtome to a thickness of 3-4 micrometers. For each tissue sample, three different depths of cut were made, which were first stained with hematoxylin-eosin (HE). Observation of HE stained sections involved determination of the form and intensity of inflammation.

Samples with insufficient tissue, as well as samples on which tissue necrosis was observed, were excluded from the study (nine samples).

**Immunohistochemical staining**

Three to four micrometer thick sections of the dental pulp were mounted on 3-aminopropyltriethoxysilane (APES) - coated slides, deparaffinised in xylene and rehydrated via graded ethanol solutions. Then sections were rinsed with distilled water and washed three times with PBS (pH 7.4). Heat-induced pretreatment for antigen retrieval (sections were immersed in a 10 mmol/L citrated buffer, pH 6.0, at 60°C for 5 min) was carried out prior to incubation with the primary antibody. The endogenous peroxidase activity was inhibited by incubation of the samples with 0.3% hydrogen peroxidase in methanol for 30 minutes at the temperature of 4°C. After blocking the non-specific reactions with 10% normal rabbit serum, the sections were incubated with the primary antibody against MMP-2 (Clone 17B11, Leica Biosystems, Novocstra, Newcastle, UK) and MMP-9 (Clone 15W2, Leica Biosystems, Novocstra, Newcastle, UK) for two hours. The sections were subsequently incubated with anti-mouse secondary antibody conjugated with avidin-biotin-peroxidase complex. The color reaction was developed using 3-3′diamino-benzidine (DAB). Finally, the sections were counterstained with Mayer’s hematoxylin, mounted and cover slipped.

**Evaluation of immunohistochemical staining**

The slides were examined by light microscopy using an Olympus BX40 microscope (Artisan Scientific, Champaign, Illinois, USA) at magnification of 400×. The product of the immunohistochemical reaction was detected in the cytoplasm of the endothelial cells, fibroblasts and inflammatory cells. MMP-2 and MMP-9 positive cells were identified by their brown color, ranging from light brown to dark brown. The cytoplasmic staining intensity was scored semi-quantitatively, according to a previously described method [15], as follows: no staining (score 0), less than 10% of stained cells (score 1), less than 30% of weakly stained or strongly but incompletely stained cells (score 2) and more than 30% of strongly and completely stained cells (score 3).

**Statistical analysis**

Comparison of MMP-2 and MMP-9 expression between healthy and inflamed dental pulp was evaluated by the Mann-Whitney U test. The statistical significance of differences between MMP-2 and MMP-9 expression in different cell populations was estimated by the chi-square test. All data analyses were carried out using the Statistical Package for Social Sciences, version 20 (SPSS Inc., Chicago, IL, USA).

**Results**

Immunohistochemical analysis revealed a significantly greater expression of MMP-9 in inflamed than in healthy dental pulps (Mann–Whitney U, p=0.0001). In contrast, there was no difference in expression of MMP-2 between these two groups (Mann–Whitney U, p=0.907). MMP-9 immunoreactivity was detected the most frequently in endothelial cells (n=33) (Figure...
1) then in inflammatory cells (n=16) and fibroblasts (n=13) (Figure 2).

Simultaneous expression of MMP-9 in endothelial cells, inflammatory cells and fibroblasts was observed in nine cases; in endothelial cells and fibroblasts in three cases; and in endothelial and inflammatory cells in six cases (Figure 3).

MMP-2 immunoreactivity in inflamed pulp was detected in endothelial cells in 11 cases and only twice in inflammatory cells (Figure 4).

As shown in Figure 5, no MMP-2 immunoreactivity was detected in the 41 tissue samples in the experimental group.

There was a statistically significant difference between the experimental and control group in the expression of MMP-9 in endothelial cells (chi-square, p=0.0001), Table 1, as well as in fibroblasts (chi-square, p= 0.0001), Table 2.

No statistically significant differences in the expression of MMP-2 in the endothelial cells of the experimental and control groups (chi-square, p=0.88) were observed, Table 1. Comparison of MMP-2 expression in fibroblasts would have made little sense due to the negligible number of positive samples.

Figure 1. MMP-9 positive endothelial cells (arrows) in inflamed pulp (IH, X400).

Figure 2. MMP-9 positive inflammatory cells and fibroblasts in inflamed pulp (IH, X400).

Figure 3. MMP-9 immunostaining localizes to endothelial and inflammatory cells in inflamed dental pulp (IH, X400).

Figure 4. Very few MMP-2—mild positive inflammatory cells (IH, X200).

Figure 5. Inflamed dental pulp with MMP-2 negative immunostaining (IH, X400).
As shown in Table 3., there was a statistically significant difference between the experimental and control group in cytoplasmic staining intensity of MMP-9 (Mann–Whitney U, p=0.0001), while was not statistically significant differences in cytoplasmic staining intensity of MMP-2 (Mann–Whitney U, p=0.907).

**DISCUSSION**

During the progression of inflammation, proteolysis of the ECM is one of the key events. In this process, the most important role is played by MMPs.

The expression of MMP-9 in inflamed dental pulp has been encompassed by several studies that used different tests. Thus, the levels of MMP-1, MMP-2, MMP-3 and MMP-9 in clinically healthy and inflamed dental pulps were determined using enzyme-linked immuno-sorbent assay (ELISA) [16]. As the overexpression of MMP-9 in inflamed dental pulp was positively correlated with gelatinolytic activity, it was concluded that MMP-9 may be a potential marker protein for assessment of the dental pulp status. Using real-time polimerase chain reaction (RT-PCR) and immunohistochemistry, expression of MMP-9 was shown to be significantly increased in inflamed in comparison to healthy pulps [17]. Although immunohistochemical expression of MMP-9 was observed in odontoblasts, fibroblasts, inflammatory and endothelial cells, the number of the pulp samples in which the mentioned cells were positive for MMP-9 was not specified. A pilot study was conducted on the basis of the aforementioned research findings. In this study, a fluorescent as-

**Table 1. Immunohistochemical expression of MMP-2 and MMP-9 in endothelial cells**

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**Table 2. Immunohistochemical expression of MMP-9 in fibroblasts**

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say was used to test the levels of MMP-9 in the dentinal fluid collected during the preparation of teeth with a clinical diagnosis of irreversible pulpitis or required replacement fillings [18]. Despite the many limitations of the study, the fact that MMP-9 was detected only in the dentinal fluid samples of the teeth with clinical diagnosis of irreversible pulpitis indicates the possible significance of MMP-9 as a potential molecular predictor in the assessment of the dental pulp condition. Comparison of gelatinolytic activity in ten healthy and ten inflamed pulp samples once again confirmed an increased level of MMP-9 in inflamed dental pulps [19]. In our research, the immunohistochemical expression of MMP-9 was significantly higher in the irreversibly inflamed than in the healthy pulp with a very high level of significance (p = 0.0001), which supports the previous findings. In inflamed pulps, MMP-9 was the most frequently detected in endothelial cells, followed by the inflammatory cells and fibroblasts. In healthy pulps, MMP-9 was detected exclusively in endothelial cells in seven cases. It is known that most of MMPs are synthesized as inactivezymogens that require activation. Several cytokines (IL-1, TNF-α) and bacterial toxins induce relief of MMPs from pulpal cells, which has been proven in cell cultures [20-23]. These data indicates the possibility that MMP-9 is deposited in the pulp cells in the form of precursors, which are released during inflammation influenced by bacterial products and inflammatory cytokines. The results of our study support the assumption that MMP-9 plays a key mediating role in the degradation of the inflamed pulp tissue.

While there are quite consistent data regarding the expression of MMP-9 in inflamed dental pulp, the respective available data for MMP-2 are rather conflicting. A study in which the quantification of matrixins was performed by ELISA concluded that the level of MMP-2 was significantly higher in inflamed than in healthy pulps [24]. The same study, which used immunohistochemistry, established that the MMP-2 was weakly expressed in inflammatory cells and fibroblasts tested groups. This is in contrast to the results of our research. Furthermore, another group of authors, who also used ELISA, points out that the level of MMP-2 was significantly lower in the group with symptomatic pulpitis than in healthy pulps [16]. In our research, there was no significant difference in the quantity and intensity of immunohistochemical expression of MMP-2 between healthy and inflamed pulps. What is interesting is that the expression of MMP-2 was almost completely restricted to the endothelium of the vasculature in both the experimental and the control group. Immunohistochemistry was used to analyze the expression of MMP-2 and MMP-9 in the pulp of teeth that are subject to orthodontic traction [25]. The analysis revealed that in a very small sample of the control group (four healthy pulp samples) the expression of the aforementioned matrixins was mainly limited to the

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endothelial cells, which is consistent with the results of our research. Given that our research showed that the expression of MMP-2 was almost identical in healthy and inflamed pulp, there remains an open question as to what its role in the dental pulp. It is known, however, that matrix metalloproteinases have numerous roles in development of both physiological and pathological conditions. The real role of MMP-2 in the pulp tissue and the reasons why its expression is restricted mainly to the endothelium of the vasculature could be an interesting subject for further studies.

**Conclusion**

The conclusions of this study are the following:

1. MMP-9 is highly overexpressed in inflamed dental pulps.
2. There are no differences in the immunohistochemical expression of MMP-2 between healthy and inflamed dental pulps.
3. Dental pulp inflammation contributes to the expression of MMP-9, while it has no influence on the expression of MMP-2.
4. Endothelial cells represent a major cellular source of MMP-9, as well as MMP-2, in healthy and inflamed human dental pulps.

**Declaration of interest**

The authors declare no conflicts of interest.

**References**


