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Expression of matrix metalloproteinases 2 and 9 in odontogenic myxoma *in vivo* and *in vitro*

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Abstract: Odontogenic myxoma is a benign neoplasm, which presents local invasiveness and tendency for recurrence. Matrix metalloproteinases (MMPs) 2 and 9 are involved in tumor invasion. Thus, the aim of this study was to analyze the expression and activity of these MMPs in odontogenic myxoma *in vivo* and *in vitro*. Three cases of odontogenic myxoma and cultured cells derived from this tumor (Mix1 cell line) were used. The detection and activity of two MMPs (2 and 9) were performed by immunohistochemistry in formalin-fixed, paraffin-embedded sections of odontogenic myxoma and immunofluorescence of the cultured cells and, by gelatin zymographic analysis of Mix1 conditioned media, respectively. MMPs 2 and 9 were detected *in vivo* and *in vitro*. The zymographic assay detected latent MMP-2 as well as latent and active MMP-9. Based on our findings, we suggest that MMPs may be involved in local invasiveness of the odontogenic myxoma. MMP-9 is not only secreted by odontogenic myxoma but also has enzyme activity with no further stimulation. Other MMPs were not analyzed; however, our results suggest that the invasive behaviour of odontogenic myxoma could be related at least to MMP-9. (J. Oral Sci. 50, 187-192, 2008)

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Introduction

Odontogenic myxoma is a locally invasive, benign neoplasm, composed of round and spindle-shaped cells lying in an abundant myxoid stroma (1). It is essentially a tumor of the jaws which usually presents as a slow growing painless mass, and is mostly detected during routine radiographic examination. Nevertheless, this lesion can show extensive bone expansion (2).

The tumor is rich in extracellular matrix (ECM) represented by type I collagen, fibronectin, tenascin, chondroitin sulfate and especially abundant in hyaluronic acid (3-5). This excessive ECM production has been implicated in the invasive behaviour of the tumor. However, the mechanisms involved in the local invasiveness of odontogenic myxoma have not been established yet.

Matrix metalloproteinases (MMPs) are zinc-dependent enzymes that can degrade structural components of the ECM facilitating the invasion of tumor cells through normal tissues (6,7). These enzymes are involved in the progression and metastasis of several tumors (8-11).

MMPs have been detected previously in odontogenic tumors. Enamelysin (MMP-20) was observed in odontomas and in tooth forming cells having an essential role in normal enamel development (12,13). Additionally, MMP-1, 2 and 9 were detected in ameloblastoma, another benign tumor with locally invasive behavior (14,15).

Considering the importance of the MMPs in degrading

bone matrix (16), this study aimed to verify the presence and activity of MMPs in odontogenic myxoma. Among the 26 types of MMPs (17,18), we chose to pursue the presence and activity of the MMPs 2 and 9, as these MMPs are mostly related to tumor invasiveness.

Materials and Methods

Sample collection

Cases of odontogenic myxoma were retrieved from the files at the Department of Oral Pathology. The specimens were obtained from three young patients (15 to 25 years old); 2 females (maxillae) and 1 male (mandible). The lesions were radiolucent and asymptomatic. The biopsy material was immersed in 10% buffered formalin solution for at least 24 hours. After washing in water, the specimens were dehydrated and embedded in paraffin. Tissue specimens were sectioned at 4 µm and stained with hematoxylin-eosin (H-E) for routine histological examination.

This research was approved by Human Research Ethical Committee of the Facudade de Odontologia, Universidade de São Paulo (project #39/06).

Immunohistochemistry

Sections at 3µm from the specimens were used for streptavidin-biotin immunohistochemical examination. Immunolabeling was carried out at room temperature. For antigen retrieval, the sections were immersed in 10 mM citric acid solution, pH 6.0, for 30 min at 95°C. For endogenous peroxidase inactivation, the tissue sections were immersed into two baths of a methanol solution containing 3% H_2O_2 for 5 min each. The tissue sections were washed during the incubation steps with Tris-HCl buffer (pH 7.4). Sections were then blocked with 1% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO, USA) in Tris-HCl to suppress non-specific binding of subsequent reagents. Then, the sections were incubated overnight with either a mouse monoclonal antibody directed against MMP-2 (Neomarker, Lab Vision, Fremont, CA, USA) or a rabbit polyclonal antibody directed against MMP-9 (Neomarker) both diluted in 1% BSA in Tris-HCl buffer (pH 7.4) at 4°C in a humidified chamber, at the concentration of 1:35 or 1:150, respectively. After incubation with the primary antibody, the sections were washed in Tris-HCl buffer and exposed to the secondary and tertiary antibodies (kit-LSAB, Dako Corporation, Carpinteria, CA, USA) for 30 min in a humidified chamber. Diaminobenzidine (Sigma Chemical Co.) was used as chromogen. Samples were then counterstained with Mayer's hematoxylin. Breast carcinoma served as positive controls, whereas the negative controls were obtained by replacing primary antibodies by 1% BSA in Tris-HCl. The observations and photographic recording were carried out using a Zeiss Axiophot II Microscope (Carl Zeiss, Oberköchen, Germany).

Cell culture

A cell line originating from an odontogenic myxoma (Mix1 cells) was used. The cells present all features of the original tumor (5). Mix1 cells grown between the eighth and the tenth passages were cultured in Dulbecco's modified Eagle medium (DMEM, Sigma Chemical Co.) supplemented by 10% fetal bovine serum (FBS, Cultilab, SP, Brazil) and 1% antibiotic-antimycotic solution (Sigma Chemical Co.). The cell line was maintained in a humidified atmosphere of 5% CO_2 in air at 37°C. The cell growth was monitored daily using phase contrast microscopy and the medium was changed every other day (19).

Immunofluorescence

Mix1 cells were plated on round glass cover slips (Ted Pella Inc., Redding, CA, USA) and maintained in serum-free DMEM, supplemented with 3µg/ml of monensin (Sigma Chemical Co.) for 24 h.

The cells were then fixed in 1% paraformaldehyde in PBS for 10 min, rinsed in PBS, permeabilized with 0.5% Triton X-100 in PBS solution for 10 min and subjected to a standard immunofluorescence protocol using the same antibodies of the immunohistochemical technique. Monoclonal primary antibodies were diluted in 1% BSA in PBS, as follows: MMP-2 (1:25) and MMP-9 (1:75). After this incubation, the cells were washed three times in PBS and then labeled with the secondary fluorescent antibodies (FITC) diluted to 1:100 in 1% BSA in PBS (Amersham Co., Arlington Heights, IL, USA). All the incubations were carried out for 60 min at room temperature and omission of the primary antibodies served as negative controls.

The observations and photographic recording were carried out with a Zeiss Axiophot II Fluorescence Microscope (Carl Zeiss).

Zymography

The culture media of confluent Mix1 cell monolayers was replaced by serum-free media overnight in order to obtain a conditioned media. This conditioned media was used for the zymographic study applying a modification of the method described by Fisher and Werb (20) to investigate the activity of these MMPs.

Conditioned medium was mixed in a proportion of 3:1 (v/v) with Laemmli buffer. The samples were subjected to SDS-PAGE with 1 mg/ml of gelatin. Gelatin is

considered the best substrate for gelatinases MMP-2 and 9 (21-23). Electrophoresis was performed for 20 min at 15 mA followed by 150 min at 20 mA. The resulting gels were washed in 50 mM Tris (pH 7.8) including 2.5% Triton X-100 solution and then were incubated for 24 h in reaction buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 5 mM $CaCl_2$) at 37°C. Finally, the gels were stained with Coomassie Blue (Sigma) for 24 h and destained with 10% acetic acid in 50% methanol for 48 h. The proteolytic activity was detected by the appearance of light bands against the dark blue background and analyzed in a computer system.

Results

Immunohistochemistry

MMP-2 was detected in all samples, and MMP-9 in only two. Figure 1 illustrates the labeling of these MMPs. MMP-2 appeared as dots throughout the whole cytoplasm of the tumor cells (A). MMP-9 in the cytoplasm was stained in a homogeneous fashion and was also detected in the ECM following the wavy direction of the ECM fibers (B).

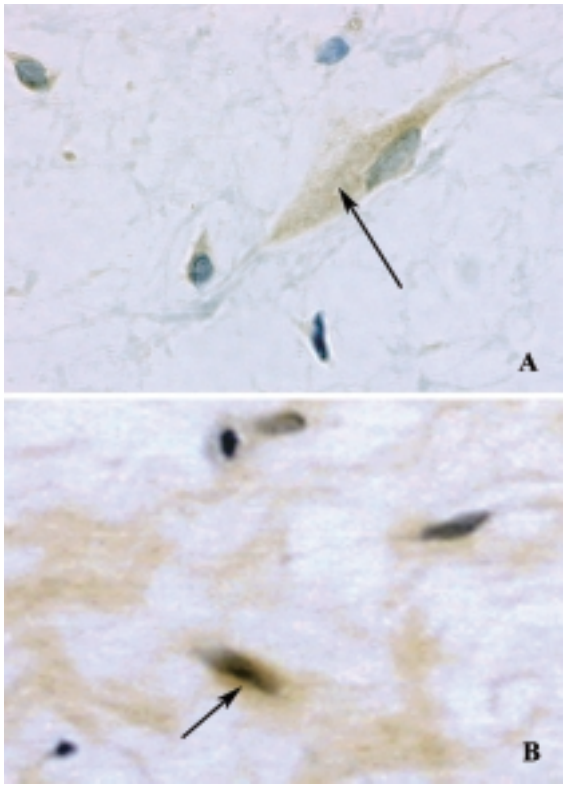

Fig. 1 Immunohistochemical reactivity for MMPs in odontogenic myxomas. MMP-2 was present as dots throughout the whole cytoplasm of the tumor cells (A, arrow). MMP-9 was stained in the cytoplasm in a homogeneous fashion (B, arrow) and was detected in the extracellular matrix (B).

Immunofluorescence

All Mix1 cells expressed MMP-2 and MMP-9 (Fig. 2). MMP-2 was observed as dots dispersed throughout the cytoplasm, with higher concentration around the nuclei (Fig. 2A). MMP-9 staining of the cytoplasm appeared as clusters of fine dots concentrated in the main cell body and dispersed at the cytoplasmic extensions (Fig. 2B).

Zymography

Three bands were detected by the zymographic analysis using gelatin as substrate (Fig. 3). Two faint bands corresponding to the latent MMP-9 (92 kDa) and active MMP-9 (84 kDa) were detected. Additionally, a strong 72 kDa band corresponding to latent MMP-2 was noted.

Discussion

In spite of being a benign tumor, the odontogenic myxoma presents a high recurrence rate of around 25% (24). Some authors relate this phenomenon to an incomplete encapsulation of the lesion or due to the capacity of the tumor cells to penetrate through bone trabeculae (25).

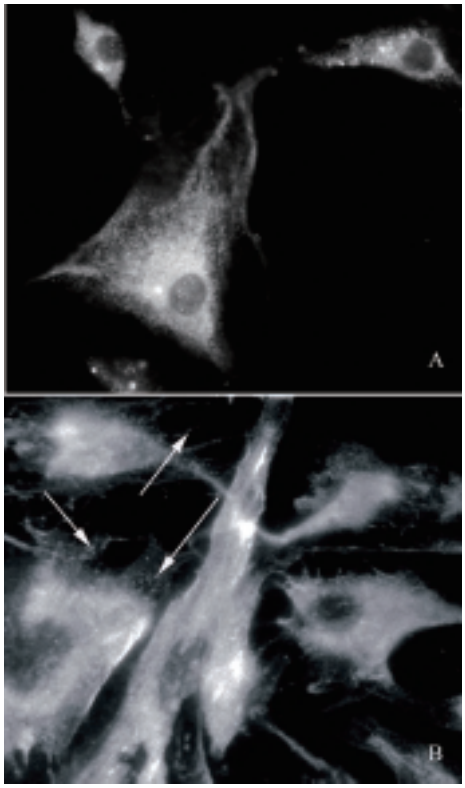

Fig. 2 Detection of MMPs in Mix1 cell line by immunofluorescence. MMP-2 was observed as dots throughout the cytoplasm with higher concentration around the nuclei (A). MMP-9 staining of the cytoplasm appeared as clusters of fine dots concentrated at the main cell body (B) and dispersed at the cytoplasmic extensions (B, arrows).

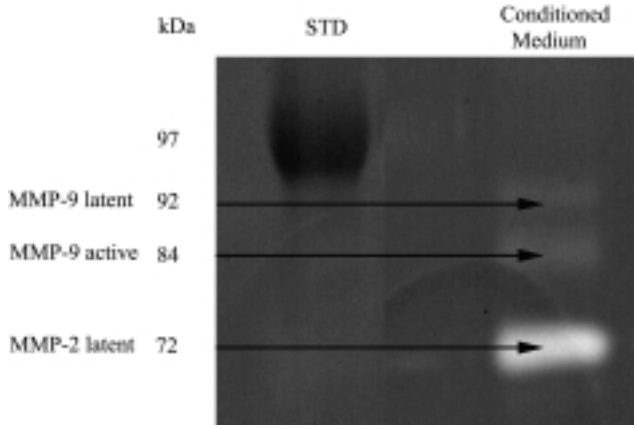

Fig. 3 Zymography showed gelatinolytic activity at molecular weights corresponding to MMP- 2 and 9. Conditioned media samples from Mix1 cells showed in the gelatin substrate, two faint bands corresponding to the latent (92 kDa) and active MMP-9 (84 kDa). Additionally, a strong 72 kDa band corresponding to the latent MMP-2 was noted.

Little is known about the relationship between the tumor cells and the extracellular matrix (ECM) or the capacity of these cells to penetrate through the bone trabeculae facilitating tumor growth. Proteolytic enzymes, such as the matrix metalloproteinases (MMPs), are frequently involved in this process (26,27).

MMPs hydrolyze components of the ECM and are synthesized by cells of the connective tissue such as fibroblasts, osteoblasts and odontoblasts (28). Under normal physiological conditions, the MMPs are expressed only when needed for tissue remodeling during angiogenesis and development. Aberrant expression is often associated with tissue destruction as in arthritis, wound healing and cancer (18,29).

In an attempt to improve our understanding of the odontogenic myxoma local invasiveness phenomenon, we searched for MMPs in tumor samples as well as in a cell line derived from odontogenic myxoma (5). We chose to detect the expression of MMP-2 and 9 because these gelatinases are frequently involved with the invasive phenotype of some lesions. This characteristic of MMP-2 and 9 is based on their ability to degrade type IV collagen, the major structural component of basement membrane as well as gelatins (30,31).

Presence of MMP-2 and 9 was observed *in vivo* and *in vitro*. In the *in vivo* samples, the label for MMP-2 was weak and restricted to the cytoplasm of the cells, whereas MMP-9 could be found inside the cells and the ECM. Although MMP-9 was not detected in all cases of odontogenic myxoma, when present the label was stronger than that of MMP-2 in the tumor sections. Moreover, the presence of MMP-9 in the ECM is of importance and could indicate high secretion of this enzyme in quantities enough to be exported and detected at the extracellular space. This result was surprising and contrary to the observations made by Bast et al. (32). Expression of MMP-9 was not observed in any of the 26 cases of their study. They reported expression of MMP-2 in 90% of their cases (32). Similar findings of MMP-2 were also observed in our study. However, these analyses were done in paraffin sections treated by immunohistochemistry, a technique with which it is only possible to observe the label of proteins with not much detail. It is possible to observe whether the label is cytoplasmic or nuclear or even whether it is present in the cells or/and in the ECM. This could also explain why in just one of the odontogenic myxoma tumors, the label for MMP-9 was not detected. It is possible that this sample had some fixation problems which made it difficult to identify non constitutive proteins, such as MMP-9.

Cell culture would be an appropriate optional technique for more detailed information regarding the presence of MMPs and also the detection of the active or latent forms of these enzymes. We decided to use Mix1 cells because *in vitro* this cell line exhibits several features of the *in vivo* tumor cells, expressing vimentin, type I collagen, fibronectin, tenascin and hyaluronic acid (5). These cells were expected to show a similar secretion pattern of MMPs to those cells *in vivo*.

As intracellular detection of MMPs *in vivo* and *in vitro* would be difficult due to their low production and quick release into the ECM, we added monensin to the culture medium for immunofluorescence analysis. This drug inhibits the extracellular transportation of these enzymes, because of its ability to destroy the Golgi apparatus without interfering with protein production (33,34).

The immunofluorescence analysis for detecting MMP-2 and 9 in the Mix1 cells confirmed the *in vivo* immunohistochemical findings. Both enzymes were detected with some differences in the labels. Mostly, these differences resided in the distribution of the enzymes in the cytoplasm of the Mix1 cells. MMP-2 was more concentrated around the nuclear region, where the Golgi apparatus is located, whereas MMP-9 was more equally distributed in the cytoplasm with a peculiar appearance at the cytoplasmic extensions as fine dispersed dots. These dots were similar to those previously described by Jaeger et al. (35) of the label of vimentin in a pleomorphic adenoma cell line. The authors explained this labeling as active transportation of this protein to the peripheral domain of the cell cytoplasm. Then, the presence of MMP-9 as isolated dots at the peripheral domain of the Mix1 cell cytoplasm could be

interpreted as active transportation of this enzyme, which in turn could explain the presence of this enzyme in the ECM *in vivo*.

The label of MMP-9 in the cells *in vivo* and *in vitro*, along with the presence of this enzyme in the ECM *in vivo*, suggested an important role of MMP-9 in odontogenic myxoma. This was corroborated in the zymographic study, where only MMP-9 was present in the conditioned media in both forms, active and latent. Recent findings indicate that pro-MMP-9 is itself a proteolytically active enzyme capable of degrading gelatin (36). Thus, this enzyme in both forms can degrade ECM. Based on these results, we can infer that MMP-9 is a potential candidate responsible for the invasive characteristic of odontogenic myxoma *in vivo*.

In the zymographic analysis using gelatin as substrate, MMP-2 was detected in the Mix1 conditioned medium; however, this enzyme was present only in the latent form. It has already been suggested that high levels of zymogens in the conditioned culture media indicates a lack of appropriate enzyme activators (37). MT1-MMP is a well known proMMP-2 enzyme activator (38-40). Thus, odontogenic myxoma may be a tumor with no appropriate MMP-2 activators, and the presence of MT1-MMP should be investigated in the future.

Our results detected in odontogenic myxoma tumors the expression and activity of some important MMPs frequently involved in the invasive process. Thus, we may infer that both MMP-2 and 9 could play a role in the invasiveness of odontogenic myxoma in the surrounding tissues. However, further studies are necessary to understand better the role of these enzymes in odontogenic myxoma invasiveness and development inside the bone.

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