Assaying endogenous matrix metalloproteinases (MMPs) in acid-etched dentinal cavity walls

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Endogenous dentinal matrix metalloproteinases (MMPs) have been implicated in the auto-degradation of collagen fibrils within resin infiltrated layers of dentinal attachment. In order to target these proteinases, we must know which MMPs are produced and activated at the resin/dentin interface. In this study, we have optimized an extraction procedure and quantitated levels of endogenous MMPs in samples of dentin removed from the cavity walls of a single, extracted tooth. In our tooth-cavity model, an occlusal cavity (2×4×2 mm) was prepared and removed from the tooth crown, leaving surrounding dentinal walls of 1-mm-thick. The samples were pulverized with an analytic mill. Using enzyme-linked immunosorbent assay (ELISA), an average of 34.7 picograms of MMP-9 was detected in less than 300 mg of dentinal powder. This is the first study of its kind to quantify endogenous levels of MMP in dentinal protein isolated from the cavity walls of a single, extracted tooth.

Keywords: Dentin, Tooth cavity model, Metalloproteinases, Hybrid layer

INTRODUCTION

Dentin bonding agents paired with the application of resin-composite materials attempt to offer optimum resin-dentin bonding and to minimize the formation of interfacial gaps by partially dissolving the dentin surface and infiltrating the porosities produced with resin. While immediate and short-term bonding to dentin have been found to be effective, a progressive decrease in bond strength after aging under in vitro and in vivo conditions has been confirmed by many studies1-3. Failure of resin/dentin attachment is expected to increase invasion of cariogenic bacteria around the restorations and promote further degradation. However, there is no conclusive clinical evidence of a relationship between the presence of bacterial infiltrated gap and breakdown of resin-bonded interfaces. Apart from bacterial enzymes, dentinal endogenous enzymes (matrix metalloproteinases or MMPs) have been suggested to contribute to the destruction of the bonded interface by auto-degradation of collagen fibrils at the resin/dentin interface and decrease long-term bonding effectiveness1,4,5.

Since endogenous dentinal MMPs are bound to collagen fibrils in mineralized dentin, the application of acidic monomers in etch-and-rinse or self-etch adhesives have been shown to be capable of releasing and activating endogenous MMPs during dentin bonding. Recent studies of whole tooth extracts have demonstrated that mineralized dentin contains MMP-2, -3, -8 and -97-9. These host-derived proteases are a group of zinc- and calcium-dependent enzymes that play strategic roles in tooth development10.

But the current hypothesis is that these same enzymes, when activated during restoration processing, can contribute to the breakdown of collagen matrices in the pathogenesis of dentinal caries11,12. These MMPs are thought to be responsible for the manifestations of thinning and disappearance of collagen fibrils from incompletely infiltrated hybrid layers in aged, bonded dentin13-18. Alternatively, these acidic resin monomers may activate latent forms of MMPs (pro-MMPs) via the cysteine-switch mechanism that exposes the catalytic domain of these enzymes that were endogenously blocked by pro-peptides19. The updated study has indicated the co-occurrence of cysteine cathepsins and MMPs in human dentin to further support the synergistic work of these two groups of enzymes20.

Use of a broad based MMP inhibitor, such as chlorhexidine, demonstrated that MMP activity in dentin can be suppressed by some protease inhibitors9, indicating that MMP inhibition may be beneficial in the preservation of the hybrid layer and resin bonding durability. In order to effectively inhibit endogenous MMP activity at the adhesive/dentin interface, identification and quantification of MMPs in dentinal cavity walls are imperative. However, earlier studies on MMP detection employed gram quantities of dentin.

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powder combined from several whole tooth samples or specific parts of dentin (e.g., deep dentin). The clinical goal is to target the detrimental, activated MMP at the resin/dentin layer without disturbing the normal stasis of the enzyme in the dentin layer. But to target the enzymes of interest, one must first know what enzymes are present and potentially activated in a single tooth. In this study, we optimized an extraction procedure in a reproducible, single tooth, cavity model that allows for measurement of endogenous MMPs in a clinically relevant sample of dentin powder obtained from the cavity walls of a single, extracted tooth.

MATERIALS AND METHODS

Dentin specimen preparation

Sound extracted non-carious human third molars were obtained from the Clinics of Department of Oral Surgery following informed consent under a protocol approved by the University of Pennsylvania School of Dental Medicine. The teeth were stored at −80°C immediately following extraction and were used in the models within 1 month.

In order to optimize detection of MMPs in a clinically relevant sample of dentin powder, three different dentin-pulverizing methods were tested in the study (Fig. 1):

1. Occlusal box type cavity

An occlusal box type cavity (2×4×2 mm) was prepared on the dentinal surface using a diamond fissure bur (SF-41, MANI, Tochigi, Japan). The coronal dentin from cavity walls was pulverized using a sterile round bar (8023 Carbide, Brasseler USA, Savannah, GA, USA) and dentin powder was collected for enzyme extraction.

2. Dentin wafer

Coronal tooth structure of teeth was sectioned to obtain 2 coronal dentin wafers of 2 mm thickness with a low speed saw (Isomet™ 1000 Precision Saw, Buehler, Lake Bluff, IL, USA) at 250 rpm/min under water-cooling. The two dentin disks were then acid etched with 38% phosphoric acid for 15 s. and pulverized with a steel hammer and pestle on dry ice to produce a fine powder. Since this method does not realistically simulate cavity preparation used in clinical restorative procedures the tooth-cavity model was developed.

3. Tooth-cavity model

Occlusal surface of the extracted tooth was removed using a low-speed diamond saw. A cavity (2×4×2 mm) was prepared on the dentinal surface by using a diamond fissure bur (SF-41, MANI). The prepared cavity was removed from the tooth crown, leaving surrounding dentinal walls of 1 mm thickness. Cavity walls were then acid-etched with 38% phosphoric acid for 15 s. and each sample was pulverized using an analytic mill with liquid nitrogen (IKA A10, IKA World, Wilmington, NC, USA), and steel mortar and pestle on dry ice to produce a fine powder.

Enzyme extraction of demineralized dentin specimens and sample conditioning

Isolated dentin powder was transferred to a 1.5 mL microcentrifuge tube and partially demineralized with 1 mL of 1% phosphoric acid (H₃PO₄) for 10 min with rotation at 4°C to increase exposure of dentin powder to the acid. All specimens were then rinsed 3 times with 1 mL of distilled water. The demineralized dentin powder was resuspended in different volumes and compositions of MMP extraction buffer (MEB; Table 1) and again rotated at 4°C for 18 h after ultrasonication with the Virsonic 50 for 3 spurts of 10 s (VirTis, Gardiner, NY, USA). The vials were centrifuged at 13,000 rpm (15,000 rcf) for 30 min at 4°C in a Beckman-Coulter Microfuge 22R Centrifuge, and the supernatants were collected. The supernatants were dialyzed through a 30-kDa cut-off membrane (Slide-A-Lyzer Dialysis Cassettes, Thermo Scientific, Rockford, IL, USA) against extraction buffer overnight. The dialyzed samples were concentrated 3× by Amicon Ultra-4 at 3,000 rpm for 10 min in a Beckman Allegra 6 centrifuge (EMD Millipore, Bellerica, MA, USA). Total protein concentration of dentin extracts was determined using Modified Lowry Assay (Thermo Scientific).

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of MMPs from dentin extracts were determined using the Human MMP-9 ELISA Kit (Life Technologies, Grand Island, NY, USA) according to the manufacturer’s instructions without dilution. Diluted standards, dentin protein extracts, and sample diluent buffer (negative control) were added in duplicate to
**Table 1** Extraction buffer composition

| Buffer | Components |
|--------|------------|
| A      | 50 mM Tris-HCl, pH 6.0, containing 5 mM CaCl₂, 100 mM NaCl, 0.1% Triton X-100, 0.1% NONIDET P-40, 0.1 mM ZnCl₂, 0.02% NaN₃, 1 mM PMSF |
| B      | 100 mM Tris-HCl, pH 7.6, containing 100 mM CaCl₂, 200 mM NaCl, 1% Triton X-100 |
| C      | 100 mM Tris-HCl, pH 7.6, containing 100 mM CaCl₂, 200 mM NaCl, 1% Triton X-100, 0.1% NONIDET P-40, 0.02% NaN₃, 1 mM PMSF |
| **D**  | 100 mM Tris-HCl pH 7.6, 0.1mM ZnCl₂, 100 mM CaCl₂, 200 mM NaCl, 1% Triton X-100, Protease inhibitor cocktail P8340 (Sigma-Aldrich) |

**RESULTS**

In the first 4 trials of this study dentinal powder was prepared from occlusal box type cavity or dentin wafer (Fig. 1-1 and 1-2). Samples ranging from 48 to 137 mg dentin were extracted in buffer A (Table 1) at a 4-fold ratio volume to weight dentin starting material. After overnight extraction at 4°C, the samples were precipitated with 85% ammonium sulfate, dialyzed in 30 kDa cut-off membrane and run on gelatin zymography as previously described. Gelatin zymography did not reveal any bands of MMP (data not shown) in these two first dentin pulverizing methods we used.

In order to better extract the MMPs from the dentin powder, a second trial was conducted with 3 samples: starting dentinal samples 103, 69 and 56 mg (Fig. 2, Lanes A–C, respectively) prepared by the dentin wafer method (Fig. 1-2). The dentin powder was extracted at a 1:1 buffer volume to weight ratio with a buffer solution containing 2-fold more Tris buffer, 10-fold more Triton X 100, 2-fold more CaCl₂, 2-fold more NaCl, with or without detergent (Buffers B and C, respectively in Table 1). In addition, the samples were dialyzed and concentrated by Amicon Ultra-4 filter centrifugation instead of ammonium sulfate precipitation (Fig. 3). With this method of concentration both Buffers B and C extractions showed bands for MMP-9 and MMP-2 confirmed by zymography and immunoblot analysis (Fig. 2).

In order to retrieve more dentin powder and optimize the extraction of MMPs, we developed the tooth-cavity model preparation (Fig. 1-3). In this model, a single tooth-cavity removed from the tooth crown was pulverized to yield 150–250 mg of dentin powder (Table 2), which was extracted in a 1:2 ratio volume to weight or 300–500 μL microplates pre-coated with respective antibodies, and incubated for 2 h. Following washing, the bonded target protein was analyzed using a biotinylated secondary antibody, developed with Avidin–Biotin–Peroxidase Complex and tetramethylbenzidine. The optical density was measured at 450 nm on a BioRad Model 680 microplate reader. Quantities of MMP9 present in the dentin extract were interpolated from an internal standard curve utilizing Prism GraphPad and associated statistical software.
Fig. 3 MMP extraction from dentin powder method optimization.
The method of MMP extraction from dentin powder was optimized by altering buffer composition and buffer ratio to starting material weight; method of dentin powder preparation; MMP detection method.

Table 2 Optimized extraction and detection of MMP-9 in milligram dentinal samples

| Extracted teeth | Dentin powder (mg extracted) | MMP-9 ELISA (pg/mL detected) | MMP-9 in dentin (pg/mg starting material) |
|----------------|------------------------------|------------------------------|------------------------------------------|
| Molar 1        | 300                          | 33.2                         | 0.02                                    |
| Molar 2        | 213                          | 30.1                         | 0.03                                    |
| Molar 3        | 252                          | 40.9                         | 0.04                                    |

Detectable levels of MMP-9 were extracted from <300 mg dentin samples prepared from the tooth-cavity model, utilizing optimized 1:2 volume Buffer D (Table 1).

The amount of endogenous MMPs present in dentinal cavity walls needs to be quantitated to effectively inhibit endogenous MMP activity at the resin adhesive interface. In this presented study, we used three different methods to optimize a pulverizing method for detection of MMPs in a clinically-relevant sample of dentin powder.

Three methods of dentin preparation were tried in this study: 1. Occlusal box type cavity; 2. Dentin wafer; 3. Tooth-cavity model. Dentin extracted by the first two methods of preparation only revealed weak bands in the gelatin zymography. Our chosen endpoint was to measure presence of MMPs, after we altered extraction buffer composition and concentration method. Previous studies of endogenous dentin MMPs had been focused on large dentin samples pooled from several extracted teeth. In these studies, the investigators were able to detect gelatinolytic/collagenolytic enzyme activities of endogenous dentin MMPs from large dentin powder samples (between 500 mg and 1 gram) pooled from several extracted teeth and internally quenched fluorescent collagen/gelatin substrate.

The failure of our first two single tooth methods suggested that our detection assay, gelatin zymography, may not have been sensitive enough for the small amounts of enzyme we were measuring. It has been confirmed that even in situ zymography has major disadvantages: the sensitivity of detection of staining intensity is rather low and therefore, this detection...
method cannot be used for quantitative purposes\textsuperscript{25}. \textit{In vitro} ELISA kits for MMPs, can detect picogram quantities of MMP \textit{versus} the nanogram quantities detected by zymogram\textsuperscript{23} and so we optimized our detection method to include ELISA.

In addition, we hypothesized that during the occlusal box type cavity prep, the pulverization of dentin in the cavity walls with burs was increasing heat at the wall and this was harmful for the MMPs. And when we pulverized dentin wafers obtained from the teeth, no heat was created but this method did not simulate a true clinical situation.

However, our third method of preparation, the tooth-cavity model, we simulate the clinical preparation scenario for a tooth restoration. Utilizing this prep method, we were successful in extracting and assaying MMPs from a single tooth-dentin sample which contains dentinal cavity walls. In addition to the change of dentin pulverization method and the use of the ELISA as a more sensitive method of detection of MMP-9, we altered buffer composition and ratio of volume to ultimately optimize extraction of MMP-9 from a single tooth’s resin/dentin layer. Ultimately, we were successful in repeatedly assaying and quantitating MMP-9 present in the dentin powder of a single tooth by utilizing the tooth-cavity model, addition of ZnCl\textsubscript{2} to the extraction buffer (buffer D in Table 1) and increasing the dentin weight:buffer volume 1:2 for extraction.

The tooth-cavity model method described in this study allowed us to quantitate MMP-9 in a clinically-relevant sample of dentin isolated from the prepared dentinal cavity walls of a single, extracted tooth. It has been confirmed that the concentrations, the distribution pattern of MMP-9 and the gelatinolytic potential of dentin matrix are variable along different dentin depths\textsuperscript{29}. It has also been shown that the predentin and dentinal tubule regions presented higher gelatinolytic activity compared to intertubular dentin. Moreover, the active sites were correlated with the distribution of MMP-2\textsuperscript{28}. Thus, differential collagen degradation potentials may be expected depending upon the depth and region in which dentin is exposed. Therefore, quantification of MMPs along dentinal cavity walls is important to obtain reliable results.

It has been confirmed that activity of MMPs can be suppressed by the use of exogenous MMP inhibitors, such as chlorhexidine, EDTA, gallardin, flavonols, sodium fluoride, tetracycline, bisphosphonates, baicalein, polyphenols and sodium trimetaphosphate (STMP)\textsuperscript{16,25,27-34}. It has also been shown that a combination of 1.5 % STMP and Ca(OH)\textsubscript{2} could also enhance the mechanical properties of demineralized dentin\textsuperscript{30}. In order to effectively inhibit endogenous MMP activity at the adhesive/dentin interface and strengthen the mechanical properties of the treated dentin, the recently presented single-tooth dentin model that contains hybrid layer of dentinal cavity walls is expected to be helpful for researchers as an innovative and reliable method of MMP detection.

CONCLUSION

A reproducible and reliable human \textit{in vitro} tooth model opens up the possibility to study targeted inhibitors of MMP activity. Our tooth-cavity model can be utilized for development of novel alternatives to chlorhexidine and the other current standards of care. This study will contribute toward assessing endogenous MMP activity in dentinal cavity walls and its inhibition, which has the potential to prolong the longevity of resin-based restorations. This method of MMP extraction from dentin can also be utilized to study other MMPs present besides MMP-9.

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