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ORIGINAL ARTICLE

Inhibition of the activity of matrix metalloproteinase 2 by triethylene glycol dimethacrylate

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Abstract The aim of this study was to evaluate the effect of different concentrations of triethylene glycol dimethacrylate (TEGDMA) on the inhibition of matrix metalloproteinase 2 (MMP-2). Mouse gingival explants were cultured overnight in DMEM and the expression of secreted enzymes was analyzed by gelatin zymography in buffers containing 5 mM $CaCl_2$ (Tris-$CaCl_2$) in 50 mM Tris-HCl buffer with the addition of TEGDMA at different concentrations (0.62%, 1.25%, 2.5%, or 5.0% (v/v)). The gelatinolytic proteinase present in the conditioned media was characterized as matrix metalloproteinase by means of specific chemical inhibition. The matrix metalloproteinases present in the conditioned media were characterized as MMP-2 by immunoprecipitation. The eletrophoretic bands were scanned and the transmittance values were analyzed. Data was plotted and submitted to linear regression to investigate MMP-2 inhibition as a function of TEGDMA concentration. Three major bands were detected in the zymographic assays. These bands were characterized as MMP-2. Zymogene (72 kDa), intermediate (66 kDa) and active forms of MMP-2 (62 kDa) were inhibited by TEGDMA in a dose-dependent way. These findings suggest that TEGDMA could inhibit MMP-2 expression even at small concentrations.

Keywords Gelatinase A · Matrix metalloproteinase 2 · Triethyleneglycol dimethacrylate · TEGDMA · Polyacrylamide gel electrophoresis

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Introduction

Triethylene glycol dimethacrylate (TEGDMA) is a cross-linking difunctional monomer present in a large quantity in dental polymeric materials, such as adhesives and composites. TEGDMA is usually used as diluent monomer due to its characteristic low viscosity. Several studies have shown that TEGDMA can be leached out of the dental resinous materials even after adequate polymerization [1–3]. This allows unpolymerized monomers to come into contact with dentin collagen after polymerization. Moreover, these residual monomers could be eluted into the oral cavity or towards dental pulp causing toxic effects [4, 5].

Actually, contemporary dental adhesives have shown satisfactory performance in short time investigations [6, 7]. However, the longevity of the adhesive restorations continues to be a great challenge to present day adhesive dentistry [7]. The combined degradation of resin and

collagen has been considered the main factor responsible for degradation of the dental bond, and consequent loss of the restoration [7]. Therefore, one of the strategies proposed for increasing the longevity of adhesive restorations has been the inhibition of dentin collagen matrix metalloproteinases (MMPs) [8].

The MMPs form a family of metal-dependent proteolytic enzymes which, collectively, are capable of degrading all types of extracellular matrix protein components, such as interstitial and basement membrane collagens, proteoglycans and fibronectin [9]. MMPs can be divided into six subgroups according to the substratum: collagenases (MMP-1 and MMP-8), stromelysins (MMP-3, MMP-10, MMP-11, and MMP-20), gelatinases or type IV collagenases (MMP-2 and MMP-9), matrilysin (MMP-7), metalloelastase (MMP-12), and membrane-type metalloproteinases (MMP-14, MMP-15, MMP-16, and MMP-17) [10]. They are secreted as inactive proenzymes (zymogens) and are thought to be activated in the tissue by cleavage of the propeptide. All members of this family have a zinc- and a calcium-binding catalytic domain, so that they depend on these ions for their activity. The *MMP* gene family in humans comprises 23 homologous proteinases, at least 20 of which have been well characterized [9]. These are widely distributed in the body and are involved in physiological and pathological processes.

Chlorhexidine (CHX) solution has been applied to the collagen mesh before application of the adhesive system and was shown to be capable of inhibiting MMPs [11–13]. Unfortunately, CHX is not copolymerizable with the methacrylate monomers being leached, which could challenge its inhibitory effect over the course of time. On the other hand, dental materials that contain zinc have also been shown to be inhibitors of human gelatinases [14–16]. However, zinc-based materials are not applicable in adhesive restorative dentistry. Recently, our research group demonstrated that 2-hydroxyethyl methacrylate (HEMA) is an inhibitor of MMP-2 [17]. This result drew our attention to TEGDMA, another monomer widely used in adhesive dentistry. Great efforts have been made in the attempt to develop MMP-inhibiting materials. These materials could be of special interest as regards the durability of adhesive restorations. Therefore, it is very important to be able to predict the inhibition potential of the monomers present in the current adhesive systems. It is well known that crown-ether functional groups are capable of forming complexes with bivalent cations [18]. Considering the molecular structure of TEGDMA with ether groups and higher flexibility, is hypothesized that a complex could occur between the ethers and the Zn^{2+} present in the catalytic domain of the MMP. Therefore, the aim of this study was to evaluate the effect of different concentrations of TEGDMA on the inhibition of MMP-2.

Materials and methods

Chemicals

TEGDMA was supplied by Esstech Inc. (Essignton, PA, USA; Milwaukee, WI, USA). Cell culture medium and reagents were obtained from GIBCO (Grand Island, NY, USA). All reagents were purchased from Sigma Chemical Co (St. Louis, MO, USA) unless otherwise noted.

Collection and preparation of MMPs

Mouse gingival tissue specimens were dissected from the palatal, lingual, and buccal sites of two mice (aged 4 weeks). This project was carried out in accordance with the principles of the Declaration of Helsinki and was approved by the Research Ethics Committee. Immediately following excision, the tissue specimens were pooled and washed in Dulbecco's Modified Eagle Medium (DMEM) containing 80 mg/ml gentamicin, at room temperature for 30 min. After washing, the specimens were incubated in DMEM containing 40 mg/ml gentamicin at 37ºC for 24 h. After this step, the gingival fragments were discarded and the cell culture-conditioned medium (DMEM) containing the secreted MMPs was frozen at −70ºC until analysis for enzyme activity.

Zymography

The proteolytic activity was examined on 10% polyacrylamide gels containing 0.05% gelatin. The conditioned medium was mixed with an equal volume of non-reducing sample buffer (2% SDS; 125 mM Tris-HCl, pH 6.8, 10% glycerol, and 0.001% bromophenol blue) and then electrophoresed. After electrophoresis, the gels were washed twice in 2% Triton X-100 for 60 min, at room temperature and then incubated at 37ºC for 24 h in 50 mM Tris-HCl buffer, at pH 7.4, containing 5 mM $CaCl_2$ (Tris-$CaCl_2$). After incubation, the gels were stained with 0.05% Coomassie Brilliant Blue G-250 (Bio Rad, Richmond, CA). The gelatinolytic activity was detected as unstained bands.

Identification of MMPs by means of specific chemical inhibition

To assess the identity of the lytic bands present in the conditioned media, parallel experiments of zymographic inhibitions were performed. Gelatin-containing gels were incubated in Tris-$CaCl_2$ buffer at 37ºC for 24 h with the addition of 0.5 mM of EDTA (Reagen, São Paulo, SP, Brazil) to inhibit lytic activities due to MMP, while 0.5 mM *N*-ethyl-maleimide (NEM) was used to inhibit activities due to serine proteinases.

Characterization of MMP-2 by immunoprecipitation

Conditioned medium was subjected to an immunoprecipitation reaction with sheep anti-human MMP-2 IgG (The Binding Site, Birmingham, England). The immunocomplexes were precipitated with protein A-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden) for 14 h at room temperature. After washing non-specific Sepharose-adsorbed material with TBS, the immunoprecipitated material was eluted with nonreducing sample buffer for 10 min at 70ºC and assayed by gelatin zymography.

Inhibition of metalloproteinase activity by TEGDMA

In order to examine the effect of different concentrations of TEGDMA on enzyme activity, a conditioned medium containing MMPs was loaded onto preparative gelatin-containing polyacrylamide gels. After electrophoresis the gels were cut into strips of approximately 1 cm, and each strip was incubated at 37ºC for 24 h in Tris-$CaCl_2$ buffer containing one of the following experimental conditions: Tris-$CaCl_2$, 0.62, 1.25, 2.5, and 5.0% (v/v) of TEGDMA. After adding the monomers to the solution, the pH was adjusted to 7.4. and then incubated at 37ºC for 24 h in 50 mM Tris-HCl buffer, containing 5 mM $CaCl_2$ (Tris-$CaCl_2$). After incubation, the gels were stained with 0.05% Coomassie Brilliant Blue G-250.

In order to quantify the relative inhibition of MMPs by different concentrations of TEGDMA, electrophoretic bands were scanned and the transmittance (the transmittance values of the zymogen, intermediate and active form were added) was analyzed with the ImageJ software (NIH, Bethesda, MD, USA). Data was plotted and submitted to linear regression to investigate MMP-2 inhibition as a function of TEGDMA concentration. Each assay was done in triplicate and was repeated at least two times.

Results

Under the assay conditions used, three major bands were detected in the zymographic assays (Fig. 1), two sharper bands with an approximate molecular mass of 62 and 72 kDa and one broader band migrating at approximately 66 kDa (Fig. 1). These enzymes were characterized as metalloproteinases, because their activities were inhibited by EDTA (Fig. 1), and NEM (a thiol-proteinase inhibitor) had no effect on the enzyme activity (Fig. 1).

Figure 1 shows that MMP-2 activities were inhibited by different concentrations of TEGDMA in a dose-dependent way. Even at low concentrations of TEGDMA, all three bands presented some degree of inhibition after incubation for 24 h in a solution containing 5 mM $CaCl_2$ (Tris-$CaCl_2$) in 50 mM Tris-HCl buffer with the addition of TEGDMA (0.62%, 1.25%, 2.5%, and 5.0%) (Fig. 1). At the two higher concentrations of TEGDMA (2.5% and 5%) the MMP inhibition was easily observed (Fig. 1).

The highest TEGDMA concentration (5%) used in this study produced a strong inhibition of MMP-2. Only the intermediate band (66 kDa) was not completely inhibited with TEGDMA (5%). It was also shown that MMP-2 inhibition can be well predicted with a linear regression model, considering TEGDMA concentration with pro-form (r^2=0.84), intermediate form (r^2=0.92), and active form of MMP-2 (r^2=0.91) (p<0.05) (Fig. 2).

Discussion

TEGDMA is a monomer extensively used in dentin-bonding agents, in addition to being an important component of adhesive restorative materials. The results of the present study showed that even at low concentrations, TEGDMA can indeed inhibit the activity of MMP-2. This can be explained, since it is well known that crown ethers can

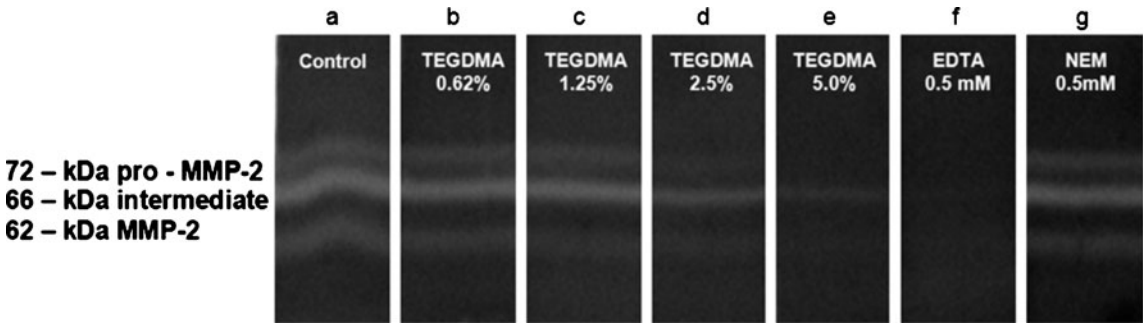

Fig. 1 Gelatin zymography showing dose response to the inhibition of MMP-2 expression by TEGDMA in conditioned Tris $CaCl_2$ buffer up to 24 h of incubation (**b**–**e**). Control lane incubated with Tris$CaCl_2$ buffer only (**a**). Characterization of the enzymes as metalloproteinases since their expressions were inhibited by EDTA and NEM had no effect on enzyme activity (**f** and **g**)

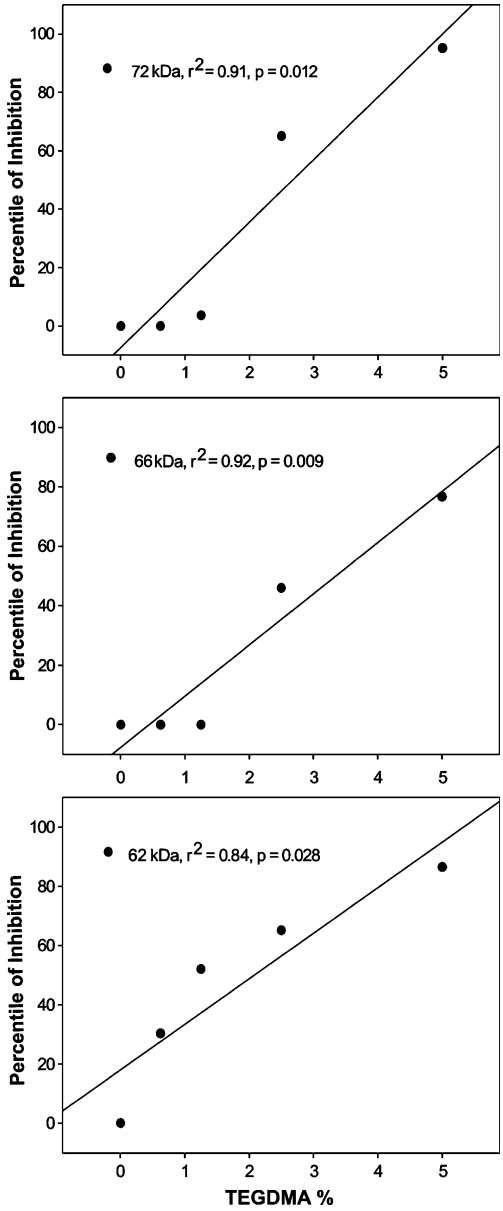

Fig. 2 Linear regression of the relationship between percentile of MMP-2 inhibition and TEGDMA%. **a** Active form of MMP-2 (r^2=0.91), **b** intermediate form (r^2=0.92) and **c** pro-form (r^2=0.84) (p<0.05)

stably form complexes with bivalent metal ions [19–21]. This suggests that the inhibition of MMP-2 produced by TEGDMA could be explained by complex formation between the ether bonds present in TEGDMA and bivalent cations Zn^{2+} present in the catalytic domain of the MMPs (Fig. 3a) or in addition, a Lewis Acid–Base Complexation interaction with the two carbonyls (Fig. 3b). Another hypothetical mechanism of inhibition that could explain the MMP-2 inhibition by TEGDMA is the reaction between carbonyl moieties of TEGDMA and the nucleophilic centers of the MMPs outside of the catalytic domain [22, 23].

As MMP-2 is the major gelatinase constituent of dentin [24] and is related to the degradation of denatured type I collagen (the major interstitial collagen in the dentin organic matrix), the use of MMP-inhibiting monomers could hypothetically improve the longevity of adhesive restorations. Some studies have suggested that MMPs play an important role in resin–dentin bond degradation [8, 11, 13]. Yet, the activation of MMP-2 and MMP-9 was also shown to play a crucial role in the destruction of dentin by caries, [25] and in the formation and remodeling of the extracellular matrix during wound healing [26]. Furthermore, these enzymes can potentiate the degradation of extracellular matrix by activating collagenase-3 (MMP-13) and neutrophil collagenase [27]. However, MMPs are known to participate in mineralization of the pre-dentin matrix, therefore, simply inhibiting these enzymes could interfere with the calcification of reparative dentin [28].

Nowadays, CHX is used in restorative dentistry with the purpose of increasing the longevity of the adhesive restorations [11–13]. This strategy consists of application of the CHX after the collagen mesh is exhibited by the acid-etching treatment of dentin. CHX may inhibit MMP-2 via the protein denaturation or cation-chelating mechanism [29]. This type of inhibition occurs according to the concentration of CHX [29]. However, it has not been determined, how durable the MMP inhibition in the CHX-treated dentin is in vivo, since CHX cannot be stabilized in the polymer matrix.

In addition to CHX, zinc oxide cements, metal salts, and dental amalgam have also been identified as MMPs

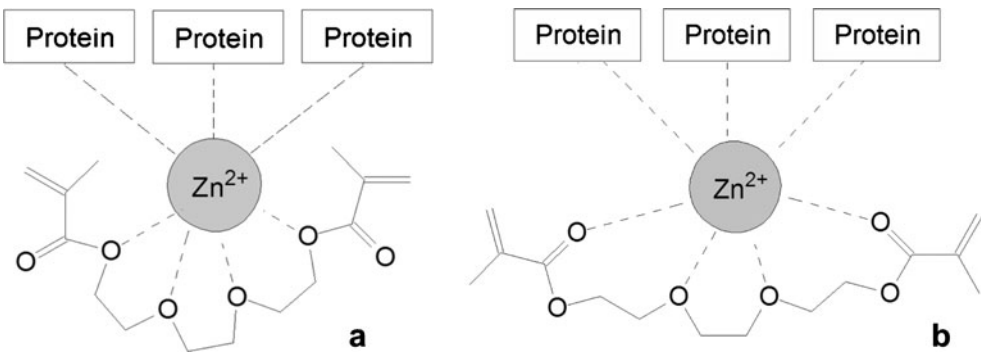

Fig. 3 Two proposed mechanisms of the zinc-dependent enzyme inhibition by TEGDMA. Complex formation between the ether bonds present in TEGDMA and bivalent cation Zn2+ (**a**) and with the two carbonyls through a Lewis type acid–base interaction (**b**)

inhibitors [14–16]. The mechanism of enzyme inactivation by metals is not completely understood. It is assumed that metal ions bind to specific sites, causing conformational changes that inactivate the catalytic function of enzymes [14]. In a recent study, it was demonstrated that HEMA, a monomer extensively used in the composition of adhesive systems, inhibited the expression of MMP-2 in vitro [17]. It was suggested that the coordination of the oxygen from a hydroxyl group with a bivalent cation Zn^{2+} could initially create a reversible inhibition of the metalloproteinase. Subsequently, proton loss could occur, leading to a covalent bond that causes an irreversible inhibition.

Previous experiments have suggested that dental adhesives are activators of gelatinolytic/collagenolytic activity in dentin [30, 31]. However, a pH-dependent activation mechanism of MMPs has also been demonstrated [25]. Thus, the acid activation mechanism of MMPs is unknown [32]. But, as the adhesive systems are acid, this could hypothetically explain why adhesives activated the MMPs in these studies. As MMPs are slowly released over time [25], the possibility of a polymerizable inhibitor that is relatively stable in the polymeric matrix can be considered an advantage. However, when an unprotected collagen layer is present, it is susceptible to proteolytic attack because the TEGDMA is immobilized; there is a limit on the inhibitory effects shown in the present study. The findings of the present study need to be carefully interpreted, as there are other components, such as acidic monomers, present in dentin adhesive systems, which can influence the effect of the total composition of the adhesive on MMP activity.

Conclusion

Our results suggest that TEGDMA could inhibit the activity of MMP-2 even at low concentrations. However, this calls for further investigations to establish the real conditions of MMP inhibition by TEGDMA in vivo.

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Conflict of Interest The authors declare that they have no conflict of interest.

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