Self-etching Increases Matrix Metalloproteinase Expression in the Dentin-Pulp Complex

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J DENT RES 2009; 88; 77

DOI: 10.1177/0022034508327925

RESEARCH REPORTS
Biomaterials & Bioengineering

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J Dent Res 88(1):77-82, January, 2009

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ABSTRACT

In adhesive restorations, one major problem is hybrid layer degradation. At present, this deterioration is explained by the activation of the endogenous matrix metalloproteinases (MMPs) present in dentin due to the acidic property of adhesive systems. We hypothesized that self-etching adhesive should also stimulate the expression of MMPs in odontoblasts. In cultured tooth slices, we evaluated the changes in MMP-2 and proMMP-9 expression in the dentin-pulp complex after self-etching adhesive treatment on dentin cavities in immunochemistry and by zymography. The treatment resulted in increased MMP-2 expression in odontoblasts, as shown by immunohistochemistry. Zymography showed increased proMMP-9 and MMP-2 in dentin under self-etching treatment when pulp was present. These results showed that self-etching adhesive stimulates the secretion of MMPs from the dentin-pulp complex and, more precisely, by odontoblasts, suggesting that odontoblasts participate in hybrid layer degradation.

KEY WORDS: odontoblast, matrix metalloproteinases, acid-etched dentin, zymography, immunohistochemistry.

DOI: 10.1177/0022034508327925

Received February 11, 2008; Last revision September 23, 2008; Accepted October 2, 2008

A supplemental appendix to this article is published electronically only at http://jdr.sagepub.com/supplemental.

INTRODUCTION

Adhesive systems are used to improve the marginal seal of a composite resin restoration at the interface between enamel and dentin. Such bonding systems require the application of acid conditioners (etch-and-rinse or self-etching adhesives) to promote the superficial demineralization of enamel and dentin (Van Meerbeek *et al*., 2003). While adhesion to enamel is stable over time, adhesion to dentin is more unstable, because of heterogeneous and wet characteristics. After superficial demineralization, the resin mixes with the dentin extracellular matrix (ECM) and polymerizes *in situ*, forming the so-called transitional 'hybrid layer'. In adhesive restorations, a significant problem is the degradation of this hybrid layer, with a loss of bond strength that affects the longevity of the restoration (De Munck *et al*., 2005; Breschi *et al*., 2008).

The major enzymes implicated in ECM degradation are the metalloproteinases (MMPs). Their structure, function, and biochemistry have been extensively documented (Visse and Nagase, 2003; Nagase *et al.,* 2006). Besides their presence in dentin tissues, the MMPs have been implicated in various physiological and physio-pathological processes during dentin-pulp complex formation and maintenance. The MMPs have been detected in soft- and hard-tissue compartments during tooth development (Heikinheimo and Salo, 1995; Bartlett and Simmer, 1999; Tjäderhane *et al*., 2002; Bourd-Boittin *et al*., 2005). The presence of MMP-2, -8, -9, -13, and -20 in intact, fully mineralized dentin (Martin-De Las Heras *et al*., 2000; Sulkala *et al*., 2004, 2007; Mazzoni *et al*., 2007) and under dentinal caries (Tjäderhane *et al*., 1998b; Chausain-Miller *et al*., 2006) has been shown.

The release and activation of endogenous proteinases have been shown to be responsible for the degradation of collagen from incompletely infiltrated hybrid layers in aged, bonded, and isolated dentin tissues *in vitro* (Pashley *et al*., 2004; Tay *et al*., 2006; Carrilho *et al*., 2007). Dentin collagen degradation at the bottom of the hybrid layer has subsequently been confirmed *in vivo* (Koshiro *et al*., 2004; Hebling *et al*., 2005). It has also been shown that etch-and-rinse (Mazzoni *et al*., 2006) and self-etching adhesives (Nishitani *et al*., 2006) can reactivate gelatinolytic activities within dentin powder, while the presence of all molecular forms of both MMP-2 and MMP-9 was recently confirmed after acidic demineralization (Mazzoni *et al.,* 2007). Most of these studies were conducted with dentin extracts and did not take into account the pulp, which, together with dentin, makes up the dentin-pulp complex.

The purpose of this study was to evaluate the possible changes in MMP-2 and MMP-9 expression in odontoblasts and pulp tissue after a self-etching adhesive treatment on dentin cavities. We used the *in vitro* thick-slice human tooth model, which offers information on the reactivity of the dentin-pulp

complex as a whole (Magloire *et al.,* 1996). We performed immunohistochemistry to identify the *in situ* localization of MMP-2 and MMP-9. We analyzed their identification and biological activity by using zymography and Western blotting procedures.

MATERIALS & METHODS

Specimen Collection

Twenty fresh, non-carious, human third molar teeth were collected with the informed consent of the donors (from 15 to 18 yrs old) and their parents, in accordance with the French Public Health Code and following a protocol approved by the local ethics committee. Immediately after extraction, the teeth were put in a BME (Basal Medium Eagle) culture medium (Gibco, Inchinnan, Scotland). We used radiography to evaluate the volume of the pulp tissue (Fig. 1, step a). An occlusal cavity was prepared in each tooth with a diamond bur (1.6 mm diameter) under water-spray cooling combined with culture medium (Fig. 1, step b). The size of this cavity was standardized, so that it did not extend over more than half the dentin thickness. Teeth were randomly assigned to two experimental groups (n = 10). A dentin bonding system (Xeno III, Dentsply De Trey, Konstanz, Germany) was applied to each cavity of the first group (Fig. 1, step c). A flowable resin composite (Ceram X, Dentsply De Trey, Konstanz, Germany) was applied to all bonded specimens. Both the dentin bonding system and the composite were used according to manufacturers' instructions, and were light-cured by means of a previously tested unit (Astralis 5, Ivoclar Vivadent, Saint-Jorioz, France). The second group, without the dentin bonding system, was the control. Teeth were then carefully sectioned in 3 or 4 slices as described previously (Magloire *et al*., 1996) (Fig. 1, step d).

Pulp-Dentin Slice Culture

Thick slices (with or without adhesive restoration and with or without pulp tissue) were cultured for 3 and 7 days as described previously (Magloire *et al*., 1996) (Fig.1). The culture medium was changed every 2 days.

Immunohistochemistry

Slices were fixed in 4% paraformaldehyde and prepared (Fig. 1-I, step f) for paraffin embedding (Fig. 1-I, step g). Microtome serial sections (5 μm) were prepared (Fig. 1-I, step h). Sections were deparaffinized, rehydrated, and incubated with anti-MMP-2 or anti-MMP-9 mouse monoclonal antibody (R&D Systems, Lille, France) (diluted 1:20 in PBS). Antibody detection was performed with Vectastain Elite ABC Kits (Vector Labs, Burlingame, CA, USA) according to the manufacturer's protocol. Negative controls were conducted with appropriate isotype-matched mouse immunoglobulins (APPENDIX).

Cell Viability Analysis

Cell viability was assessed by 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium (MTT) assay. Slices were washed twice with PBS, and a 1 mg/mL MTT solution was added for 2 hrs at 37°C in 5% CO_2 atmosphere. The MTT solution was removed, and the converted dye was solubilized in acidic isopropanol (0.04 M HCl in absolute isopropanol). The absorbance of the converted dye was measured at a wavelength of 550 nm.

Zymography

Gelatinolytic activities from conditioned media of pulp tissue cultures (Fig. 1-II, step j) or from dentin were assessed by gelatin zymography. Slices with or without pulp were washed 3x with PBS/penicillin-streptomycin and serum-starved for 24 hrs prior to being harvested (Fig. 1-III, step k). At indicated times, conditioned media were collected. MMPs from dentin were extracted by passive diffusion overnight at 4°C in Laemmli buffer (0.5 M Tris/HCl, pH 6.8; 10% SDS; 20% saccharose, bromophenol blue) at a concentration of 200 mg dentin/mL Laemmli buffer (Fig. 1-III, step m). For conditioned media loadings, volumes corresponded to an equal metabolic activity within pulp cells, determined by the MTT assay (Fig. 1-II, step i). Proteolytic activity was assayed with gelatin gels as previously described (Ntayi *et al.,* 2004). We loaded 0.1 and 0.5 ng of recombinant human proMMP-2 and proMMP-9, respectively, as standards (Calbiochem, VWR International SAS, Fontenay sous Bois, France).

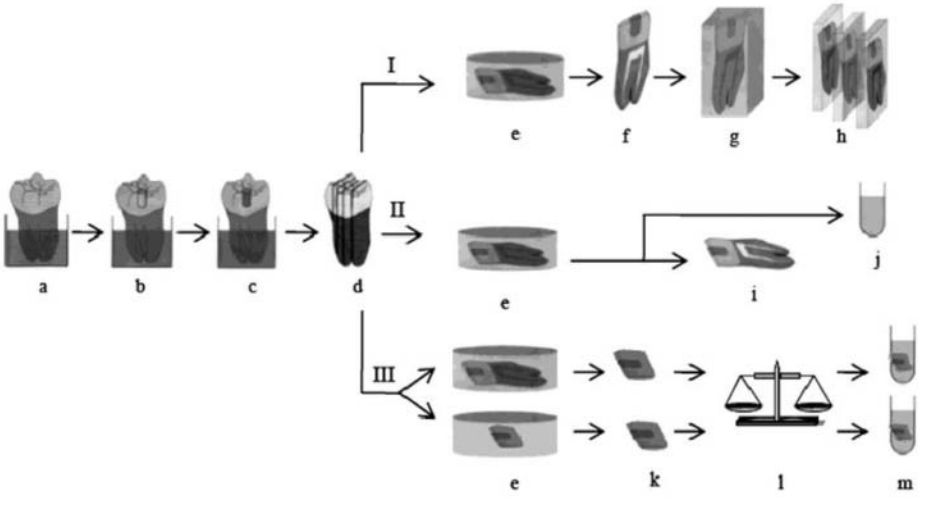

Figure 1. Diagram summarizing tooth sample treatment procedures. **(I)** Immunohistochemistry. **(II)** Zymography on culture growth medium.**(III)** Zymography on sliced teeth. **(a)** Radiography. **(b)** Cavity was prepared under water. **(c)** Teeth were randomly assigned to 2 experimental groups (with and without a dentin bonding system). **(d)** Sections about 750 μm thick. **(e)** Slices were cultured in growth medium. **(f)** Slices were fixed in paraformaldehyde and demineralized in acetic acid. **(g)** Slices were embedded in paraffin. **(h)** Microtome serial sections were collected and treated for immunohistochemical analysis. **(i)** MTT assay on sliced teeth. **(j)** Zymographic assay on culture growth medium. **(k)** Dentin crown section. **(l)** Weighing dentin crown. **(m)** Diffusion in Laemmli buffer and zymographic assay.

Western Blotting

We carried out a Western blot on conditioned media to identify the different forms of MMPs, using antibodies raised against human MMP-2 (Biodesign Int., Saco, ME, USA) or human MMP-9 (R&D Systems). Immunoreactivity was visualized in a chemiluminescent system (Pierce, Perbio Science France SAS, Brebières, France).

Densitometric Analyses

Gelatinase activity as detected by zymography was semi-quantified by densitometric analysis with "Quantity One" software (Bio-Rad, Marnes-La-Coquette, France). Background was subtracted, and the proMMP-9 standard activity (0.5 ng) was used as internal control.

Statistical Analysis

Results were expressed as mean values ± standard error obtained from 3 independent experiments (three different donors). Statistical analysis was determined with the Student's *t* test.

RESULTS

Immunohistochemical Analysis of Thick-sliced Human Teeth

On untreated tooth sections, a diffuse labeling of MMP-2 was observed associated with pulp ECM and many pulp cells (Fig. 2A). A more intense staining was visible in odontoblasts, including their processes, and in predentin. This pattern of expression was the same whichever the time of culture (day 0, day 3, and day 7) (Figs. 2A-2C). In contrast, the self-etching bonding treatment resulted in a much more intense staining for MMP-2 in the odontoblast layer and predentin after 3 days of culture (Fig. 2E). This staining was even more intense at day 7 (Fig. 2F). The treated sample at day 0 (Fig. 2D) was similar to the untreated one (Fig. 2A). For MMP-9, on untreated teeth (at day 0, day 3, day 7), the staining appeared mainly associated with pulp cells and odontoblasts, with a faint labeling of the predentin tissue (Figs. 2G-I). On treated teeth, at day 0 (Fig. 2J), the staining was similar to that in the untreated specimen. At day 3 or day 7, no striking variation could be observed (Figs. 2K, 2L).

Detection of Gelatinases Isoforms and Their Gelatinolytic Activities

Three main forms of gelatinases were detected by zymography in all samples analyzed. The enzymes migrated with apparent molecular mass, *Mr,* of 92 kDa, 72 kDa, and 64 kDa, characteristic of the latent MMP-9, the latent MMP-2, and the active MMP-2 isoforms, respectively (Figs. 3A, 3B), as confirmed by Western blot (Fig. 3C). No isoform corresponding to the activated MMP-9 (82 kDa) was detected with our procedures.

The activity of MMPs was first analyzed from conditioned media of tooth slices by zymography (Fig. 3A). Whichever of the samples was analyzed, proMMP-9, proMMP-2, and active MMP-2 were identified after 3 days or 7 days of culture. Zymograms showed an increased presence of the 3 isoforms when samples were treated with adhesive (Fig. 3A, lanes d and e), in comparison with samples without treatment (Fig. 3A, lanes b and c). Densitometric analysis of the zymograms indicated that this increase was statistically significant after 3 days of culture for proMMP-2 (about 10.55-fold), and after 7 days of culture for all 3 isoforms (4.11-, 9.42-, and 5.25-fold average increase for proMMP-9, proMMP-2, and MMP-2, respectively) (Fig. 4A).

The gelatinase activities were also evaluated from dentin extracts of slices cultured with or without pulp tissue. The extracts obtained from the tooth slices cultured without pulp displayed neither proMMP-9 nor proMMP-2 (Fig. 3B, lanes b and c), and the adhesive treatment had no apparent effect on these isoforms (Fig. 3B, lanes d and e). When the pulp-dentin complex was cultured as a whole, an increased presence of MMP was observed by zymography in dentin (Fig. 3B, lanes f and g), as compared with that in the dentin specimen alone (Fig. 3B, lanes b and c). The adhesive treatment enhanced these 3 activities (Fig. 3B, lanes h and i), as compared with that in untreated samples (Fig. 3B, lanes f and g). It resulted in an average 1.83-fold increase at 7 days of culture for MMP-2, as compared with untreated tooth slices (Fig. 4B).

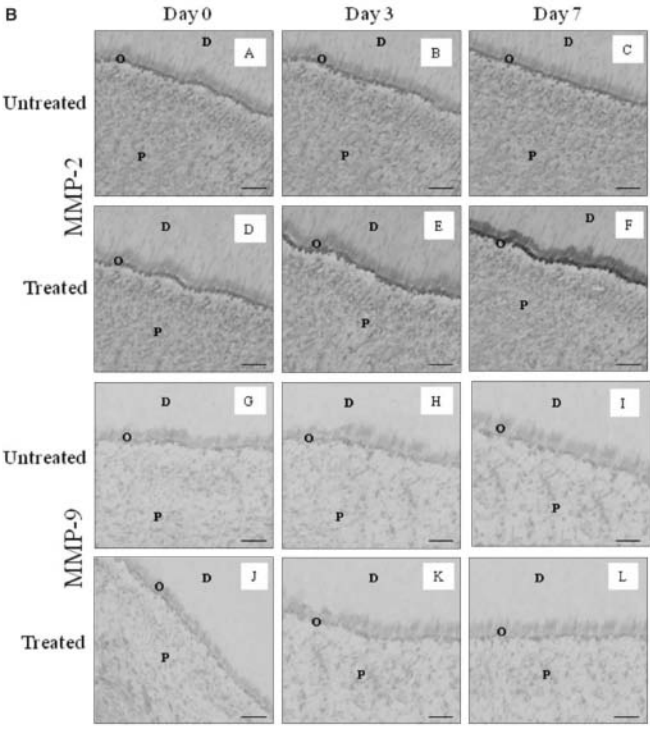

Figure 2. Immunoperoxidase staining of MMP-2 and MMP-9 in the *in vitro* thick-sliced human tooth model treated or not with a self-etching adhesive system. On untreated teeth at D_0 of culture, immunodetection of MMP-2 showed a diffuse association of the enzyme with the pulp extracellular matrix and its expression in many pulp cells **(A).** The same pattern of immunodetection was obtained at D_3 **(B)** and D_7 **(C),** and on treated teeth at D_0 **(D).** On sections treated with a self-etching adhesive system, a more intense staining was observed in odontoblasts, including their processes, and in predentin at D_3 **(E)** and D_7 **(F).** Immunodetection of MMP-9 of untreated cultured teeth at D_0 **(G),** D_3 **(H),** and D_7 **(I)** was observed in pulp cells and odontoblasts. A similar pattern was obtained at D_0 **(J),** D_3 **(K),** and D_7 **(L)** on treated teeth. Negative controls treated with appropriate isotype-matched mouse immunoglobulins revealed no staining (see Appendix Fig.). O, odontoblastic layer; P, pulp core; D, dentin. Bars: 30 μm.

DISCUSSION

In this work, we present the first evidence that self-etching adhesive treatment results in an increased presence of proMMP-9, proMMP-2, and MMP-2 in the dentin-pulp complex and indicate increased activity, at least for MMP-2, for 7 days of treatment. Such effects correlated with the significant overexpression of MMP-2 observed in odontoblasts at the tissue level.

At the dentin level, our results demonstrated the presence of the MMP-2 active form without any other gelatinases identified. This is in agreement with previous results describing the presence of active MMP-2 in healthy human dentin (Martin-De Las Heras *et al*., 2000). However, MMP-9 has previously been detected in sound dentin (Mazzoni *et al.,* 2007) after demineralization and protein precipitation, but at a two-fold lower concentration than

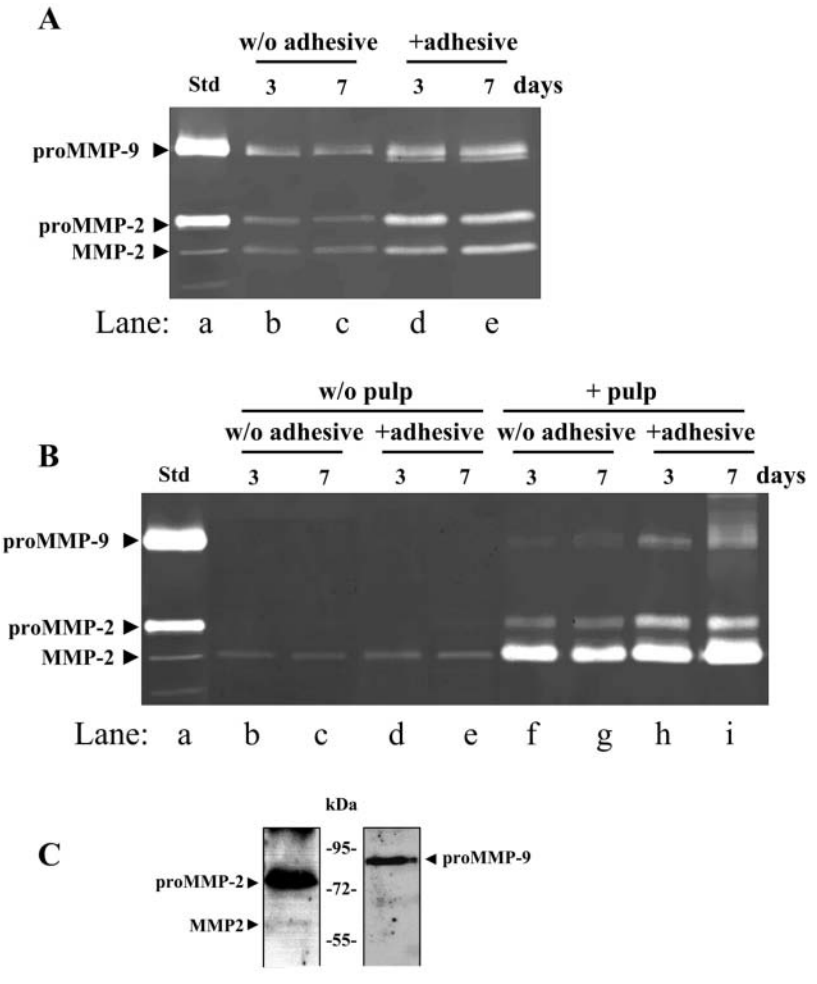

Figure 3. Zymographic analysis of MMPs. **(A)** Zymographic analysis of culture growth media. The pattern of expression is representative of three different donors. **(a)** Recombinant human proMMP-2 and proMMP-9 were loaded as standards. **(b,c)** Culture growth media from untreated tooth slices after 3 and 7 days, respectively. **(d,e)** Culture growth media from tooth slices treated with adhesive for 3 and 7 days. **(B)** Zymographic analysis of MMPs in a dentin slice specimen. The pattern of expression is representative of three different donors. **(a)** Recombinant human proMMP-2 and proMMP-9 were loaded as standards. **(b-e)** Dentin slice cultures without pulp in untreated **(b,c)** or treated **(d,e)** conditions for 3 **(b,d)** or 7 **(c,e)** days. **(f-i)** Dentin slice cultures with pulp in untreated **(f,g)** or treated **(h,i)** conditions for 3 **(f,h)** or 7 **(g,i)** days. **(C)** Identification of MMP isoforms from culture media by Western blotting with antibodies raised against the MMP-2 or MMP-9 proteins.

MMP-2. This could explain why we did not detect any MMP-9 activity by our procedure.

It is well-known that dentin tissue itself can be a source of resident proteinases such as MMP-1, -2, -9, and -20 (Chaussain-Miller *et al.*, 2006), the release and activation of which could be induced by the low pH of self-etching products (Mazzoni *et al.*, 2006; Nishitani *et al.*, 2006), since low pH has previously been shown to activate salivary MMPs (Tjäderhane *et al.*, 1998a; Sulkala *et al.*, 2001). Indeed, this "acid activation" of proteolytic activities by adhesives has been shown to correlate with their pH values (Nishitani *et al.*, 2006). However, our self-etching treatment had no statistically significant effect on MMP-2 activity from the dentin tissue. This result seems to contradict previous reports that showed a huge activation of collagenolytic and gelatinolytic activities (Nishitani *et al.*, 2006; Tay *et al.*, 2006). This discrepancy may be explained by the fact that those investigators obtained this activation on dentin powder mixed with the self-etching adhesive, where the liberation of gelatinolytic and collagenolytic activities might be more effective. Our model is closer to biological reality, with the use of the adhesive as in clinics and the ability of the pulp to respond to inductive signals or therapeutic treatments (Melin *et al.*, 2000). However, we cannot exclude the possibility that, with a larger number of samples, we would have observed a significant increase.

Dentin is not isolated in teeth, but is a part of the dentin-pulp complex. At this level, zymography of conditioned media of treated pulp-dentin cultures showed a significant increase in proMMP-9 and MMP-2. These results indicated that the pulp cells were stimulated by the adhesive to release these MMPs. This was confirmed by the fact that these activities were also present in dentin of the cultured dentin-pulp complex, compared with dentin alone. Immunochemistry highlighted the role of odontoblasts in this process for MMP-2. This is the first report of an expression activation of MMP in odontoblasts by self-etching treatment, and raises the question of how such activation is induced. Two hypotheses may be proposed. The first has already been used to explain reactionary dentinogenesis under caries lesions. The acidic demineralization of dentin by self-etching treatment could release some sequestered growth factors, and these molecules, after diffusion through the dentin tubules, could stimulate the expression of MMPs by odontoblasts. In fact, several studies have demonstrated the regulation of MMP expression by TGF-β1 and BMPs (Palosaari *et al.*, 2002, 2003) and the stimulation of MMP-2 by TGF-β1 (Tjäderhane *et al.*, 1998a) in odontoblasts. The second hypothesis implies that residual unpolymerized acidic monomers could also diffuse through tubules and create an acidic environment for odontoblasts. It has been previously shown that human odontoblasts express various ion channels, including a pH-sensitive one: TREK-1 (Magloire *et al.*, 2003). Therefore, one can speculate that the activation of such a channel could regulate MMP expression in odontoblasts. However, this hypothesis remains to be tested.

In adhesive restorations, a significant problem is the rapid degradation of the hybrid layer. Until now, this degradation was mainly explained by the activation of endogenous MMPs following self-etching treatment. However, the results of our study indicate that, even if resident proteinases are part of this process, another source of MMPs must be taken into account: the odontoblasts. Therefore, the future challenge for adhesive

restorations would be to incorporate not only effective, non-toxic inhibitors of these MMPs, but also some molecules blocking their expression by odontoblasts into restorative materials.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the staff of the Stomatology Departments (Saint Joseph Hospital and Lyon-Sud Hospital) for collecting tooth samples, and Dentsply De Trey for providing the dentin bonding system. We thank the Centre Commun d'Imagerie Laennec (Lyon) for providing technical facilities.

This investigation was supported by the Région Rhône-Alpes and the European Community (LSHM-CT-2005-018960). Romain Debret was the recipient of a post-doctoral fellowship from the European Community.

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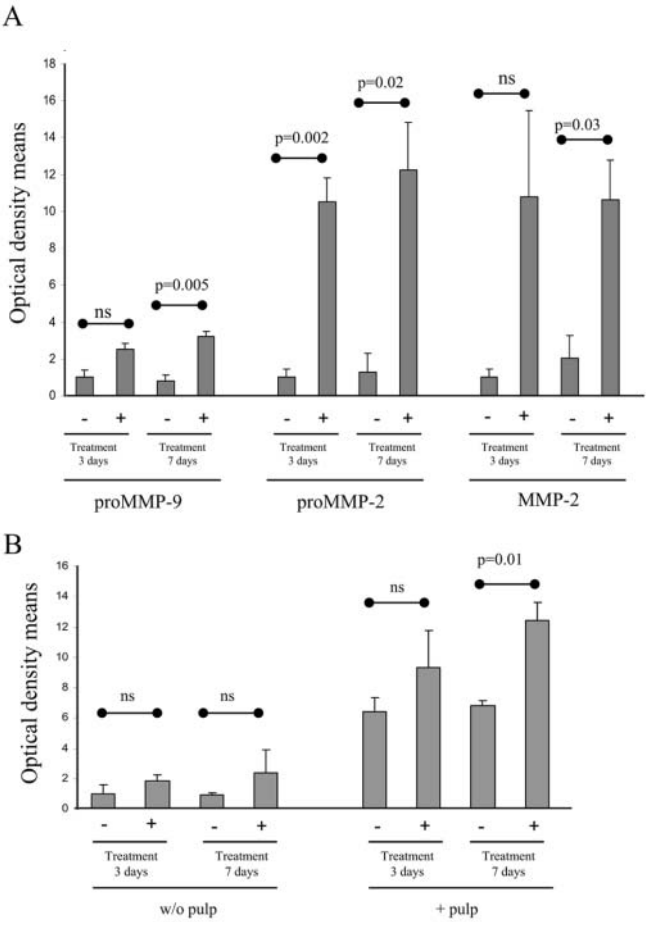

Figure 4. Densitometric analyses of zymographic gels. Densitometric values of gelatinolytic activities are expressed as optical density means (OD means) normalized to untreated samples at 3 days (Fig. 3B, lanes b). Data represent the mean OD for three donors ± SEM ($p < 0.05$). n.s. = non-significant. **(A)** Gelatinolytic activities of culture media normalized to the conditioned untreated media, at 3 and 7days for each isoform (proMMP-9, proMMP-2, and MMP-2). N = 3. **(B)** MMP-2 gelatinolytic activities in dentin extracts normalized to the conditioned untreated dentin, with and without pulp, at 3 and 7 days of culture. N = 3.

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