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MMP-2 and MMP-9 are Differentially Involved in Molar Growth

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Matrix metalloproteinases (MMPs) have been implicated in tissue development and re-modeling. Dynamic morphological changes of tooth germs reflect involvement of these enzymes during odontogenesis. The present study was performed to investigate expression and localization of MMP-2 and MMP-9, which have been known to have type IV collagenase activities, in rat tooth germs at different developmental stages. MMP-2 expression was increased gradually in the tooth germs from cap to crown staged germs at both transcription and translation levels. The localization of this molecule was detected in secretory ameloblasts and preameloblasts. The strong immunoreactivities were occasionally seen along the basement membrane between ameloblasts (or preameloblasts) and odontoblasts (preodontoblasts). However, weak reactivity was detected in odontoblasts and reduced enamel epithelium. The level of MMP-9 expression in the tooth germs was higher in cap stage than in crown staged germs at both transcription and translation levels. They were strongly expressed in both ameloblasts and odontoblasts. Even though reduced enamel epithelium after enamel formation and inner enamel epithelium at the cap stage exhibited weak reactivity, strong reactivity was detected in dental follicles and perifollicular tissues surrounding cap staged germs. These results suggested that MMP-2 may involve degradation of the basement membrane during hard tissue formation, whereas MMP-9 might be involved in remodeling of follicular tissues.

Key words : MMP-2, MMP-9, Molar

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Introduction

The tooth develops from oral ectoderm-derived epithelium and neural crest-derived ectomesenchyme. The first sign of tooth development is the formation of the dental lamina, a local thickening of dental epithelium which invaginates into the ectomesenchyme. Epithelial cells at the distal end of the dental lamina further proliferate, forming the enamel or dental organ. Tooth development is morphologically divided into the bud, cap and bell stages, depending on the shape of the enamel organ. The dentin and enamel matrices firstly begin to secrete during the late bell stage, forming the crown-staged tooth germ, which is further developed into the root-staged tooth germ. The dental ectomesenchymal cells histodifferentiate into odontoblasts, whereas inner enamel epithelia, a part of the enamel organ, differentiate into ameloblasts.

Dental morphogenesis is accompanied by changes in cellular organization and remodeling of extracellular matrix. Tissue remodeling occurs mainly by the action of matrix proteases and therefore protease investigation has been a critical issue for tooth development. Proteases have been classified into 4 subgroups: the matrix metalloproteinases (MMPs), the cystein-, aspartate- and serine dependent-proteinases (Barrett, 1994). Among these, MMPs in particular, play a crucial role in tissue remodeling by taking part in breakdown of extracellular matrix proteins and other ground substances, such as collagens and proteoglycans (Moldovan *et al*., 1997; Sternlicht and Werb, 2001; Hannas *et al*., 2007). Moreover, MMPs are implicated in both differentiation and migration of cells and programmed cell death which occur during tissue morphogenetic processes (Werb and Chin, 1998; Ortega et al., 2003, 2004; Nagase *et al*., 2006) and thus they have been regarded as key regulators in tissue development (Ortega *et al*., 2003; Park *et al*. 2009). Majority of them are inducibly expressed and regulated at both the levels of transcription and post-translation.

Thus far, at least 23 different MMPs have been identified and classified into several groups: collagenases, gelatinases, stromelysins and membrane-type MMPs (Visse and Nagase, 2003). Among these, MMP-9 (gelatinase B) and MMP-2 (gelatinase A) are important, since they have substrate preferences to denatured collagen and gelatin, as well as basement membrane type IV and V collagens (Martignetti *et al*., 2001; Janusz *et al*., 2004). Odontogenesis is accompanied by reciprocal interactions between epithelium and mesenchymal tissue (Heikinheimo and Salo, 1995; Sahlberg *et al*., 1999; Palosaari *et al*., 2000; Randall and Hall, 2002; Goldberg *et al*., 2003; Chung *et al*., 2009). Because the basement membrane intervenes between ameloblasts/preameloblasts and abutting odontoblasts/preodontoblasts, this structure should be firstly eliminated somehow. Similarly, in predentin near the mineralization front, matrix substances, such as enamel matrix proteins and proteoglycans, should be removed for mineralization of the dentin and the enamel. In developing tooth, all this enzymatic breakdown of substances in extracellular matrix can affect dental hard tissue formation, including mineralization. Degradation of these structures is primarily taken place by matrix destructing enzymes. In fact, many matrix enzymes for tooth development have been reported. MMP-2, MMP-8, MMP-9 and stromelysin-1 (MMP-3) were demonstrated during dentin and enamel formation (Hall *et al*., 1999). Enamelysin (MMP-20) could be found in the forming enamel (Bartlett and Simmer, 1999; Sulkala *et al*., 2002). But all these reports were confined within a specific developmental stage of the tooth, and were not giving intensive information for the MMPs involved. Also, specific role of each MMP for tooth development was not established yet and is still controversial.

The present study aimed at investigating temporal and spatial distribution patterns of MMP-2 and MMP-9 and elucidating their functions in the developing tooth germs.

Material and Method

Removal of tooth germs

Sprague-Dawley rat pups were used for the present study. From the preliminary histological study, the upper 2nd and 3rd molars were at the root stage and cap stages in tooth development at postnatal day 10, respectively. Moreover, the upper 2nd molars were at the early bell, crown and root stages at postnatal days 4, 7 and 10, respectively. Rat pups at postnatal days 4, 7 and 10 were euthanized. After overlying gingivae and alveolar bone were carefully removed using fine forceps, the upper 2nd and 3rd molar germs were harvested, together with follicular tissues.

Preparation for tissue sections

Portions of the maxilla containing tooth germs were immersion-fixed in 4% paraformaldehyde solution overnight. They were decalcified with ethylene diamine tetra-acetic acid (pH 7.4) over several days to weeks, and routinely processed for paraffin embedding. Five-μm-thick sagittal sections were cut and stained with H-E for morphological observations.

RT-PCR

The extracted tooth germs were immediately frozen in liquid nitrogen for total RNA extraction. The extraction was performed using a Trizol® Reagent (Gibco BRL, MD, USA). Before PCR was done, contaminated DNA, if any, was removed by treating extracted DNA with DNAse I (Gibco BRL, MD, USA). MMP-2 primer sequences were 5' GAG ATC TGC AAA CAG GAC AT 3' forward and 5' GGT TCT CCA GCT TCA GGT AA 3' reverse (GenBank accession No. NM 031054), generating an expected PCR product of 528 bp. MMP-9 primer sequences were 5' CGG TAT TGG AAG TTC TCG AAT CAC 3' forward and 5' CAC ACG CCA GAA GTA TTT GTC ATG 3' reverse (GenBank accession No. NM 031055), generating an expected PCR product of 434 bp. The house keeping gene, GAPDH (GenBank accession No. AF 106860) was amplified using primers, which had sequences of 5' CCA TGG AGA AGG CTG GGG 3' forward and 5' CAA AGT TGT CAT GGA TGA CC 3' reverse, generating an expected product of 195 bp. RNA samples were quantitated using UV spectrophotometer and qualified by obtaining OD 260/280 ratios > 1.8. AccPower RT PreMix (Bioneer, Daejeon, Korea) was used for reverse transcription. Briefly describing the PCR, mixtures of total RNA and Oligo dT_{18} were added to AccPower RT PreMix tube (Bioneer, Daejeon, Korea). cDNA synthesis was performed by incubating these mixtures at 42°C for 60 min. For PCR reaction, AccPower PCR PreMix (Bioneer, Daejeon, Korea) was used. Briefly, after cDNA and primers were added to AccPower PCR PreMix, PCR was performed in a Perkin-Elmer GeneAmp PCR system 2400 (Applied Biosystems/Perkin Elmer, Foster City, CA, USA) with the following profile: denaturation for 1 min at 95°C, annealing for 1 min at 60°C and 1 min extension step at 72°C. Preliminary experiments were performed to determine the optimum number of PCR cycles. Products were resolved on 1% agarose gel and visualized using ethidium bromide. The product size was confirmed using 100 bp (Takara, Otsu, Shiga, Japan). DNA template was omitted for the negative control in PCR.

Western blot

Ready prep protein extraction kit (Bio-RAD, Hercules, CA, USA) was used to extract protein from tooth germs. Briefly, extracted molar germs were resuspended and incubated in protein extraction solution with proteinase inhibitor cocktail (Roche, Mannheim, Germany) for 1 hr. Concentration of protein lysates was measured by using Amersham GeneQuant Pro (Amersham-Pharmacia Biotech, Arlington Heights, IL, USA). Lysates were boiled for 10 min in 3 × SDS sample buffer, loaded onto 10% continuous gradient SDS-polyacrylamide gel and then transferred to Protran

nitrocellulose membrane (Whatman GmbH, Dassel, Germany). The membrane was blocked with TBS-T buffer [10 mM Tris-buffered isotonic saline (pH 7.0), 0.1% merthiolate, 0.1% Tween-20] containing 5% skim milk for 1 hr at room temperature with shaking. The membrane was then incubated with purified rabbit polyclonal antibodies for MMP-2 and MMP-9 (Santa Cruz Biotech, Delaware, CA, USA) and purified mouse monoclonal antibody for β-actin (Sigma-Aldrich Co., ST Louis, MO, USA) overnight at 4°C with gentle shaking. Washed with TBST for 10 min, the membrane was incubated with 1:3000 anti-goat IgG horseradish peroxidase-conjugated and anti-mouse IgG horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA, USA) for 2 hr. Bound antibodies to MMP-2 and MMP-9 were visualized with Lumiglo reagent (Cell signalling, Beverly, MA, USA).

Immunofluorescent staining

Sections were deparaffinized with xylene and rinsed in PBS. Purified rabbit polyclonal antibodies for MMP-2 and MMP-9 (Santa Cruz Biotech, Delaware, CA, USA) were used. Normal serum was substituted with primary antibodies for the negative control. Immunofluorescent staining was performed using TSA™ Kit (Invitrogen, Carlsbad, CA, USA). Briefly, after blocking endogenous peroxidase by 1% H_2O_2 for 1 hr, sections were reacted with the primary antibodies overnight, and subsequently in HRP-conjugated secondary antibody for 1 hr. Finally, they were then incubated in Tyramide working solution for 10 min. Reactants were visualized and photographed using the LSM confocal microscope (Carl Zeiss, Germany).

Results

Histological findings

The upper 3rd and 2nd molar germs were at the cap and root stages, respectively, in tooth development at postnatal day 10, respectively. The cap-staged molar germs were composed of the enamel organ, dental papilla and dental sac or dental follicle surrounding the papilla. The cap-shaped enamel organ consisted of inner and outer enamel epithelia and stellate reticulum between them. The ectomesenchymal cells forming the dental papilla inferior to the enamel organ were surrounded with the dental follicle (Fig. 1). The root staged germs showed the epithelial diaphragm. The future crown showed reduced enamel epithelium and ameloblasts for enamel formation, odontoblasts for dentin formation and pulpal cells. The upper 2nd molars were at early bell, crown and root stages at postnatal days 4, 7 and 10, respectively.

Analysis of MMP mRNA expression

RT-PCR was performed using specific primers for MMP-2 and MMP-9 genes between the cap-staged 3rd and root-staged 2nd molar germs. Amplicons of the expected size 528 bp and 434 bp for MMP-2 and MMP-9, respectively, were generated from both the upper 3rd and 2nd molars, respectively. The expression level of MMP-2 was higher in the 2nd molars than in the 3rd molars, whereas that of MMP-9 was reversed (Fig. 2).

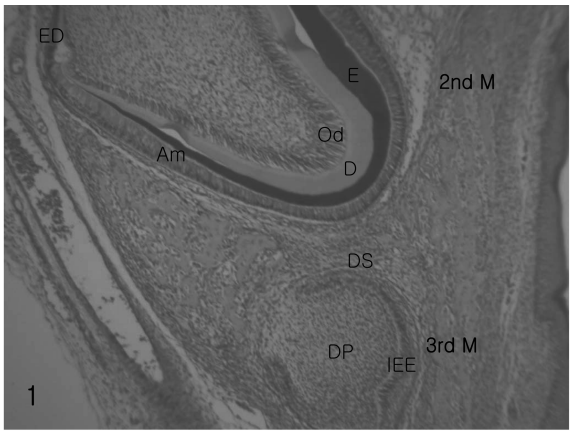

Fig. 1. The cap-staged and root-staged upper 3rd (3rd M) and 2nd molar germs (2nd M) at postnatal day 10. The cap-staged germ shows dental papilla (DP), dental sac (DS) and inner enamel epithelium (IEE), whereas the root-staged germ shows the epithelial diaphragm (ED) enamel (E)-forming ameloblasts and dentin-forming odontoblasts (Od). Am : ameloblasts, D : dentin. Magnification × 60, H-E staining.

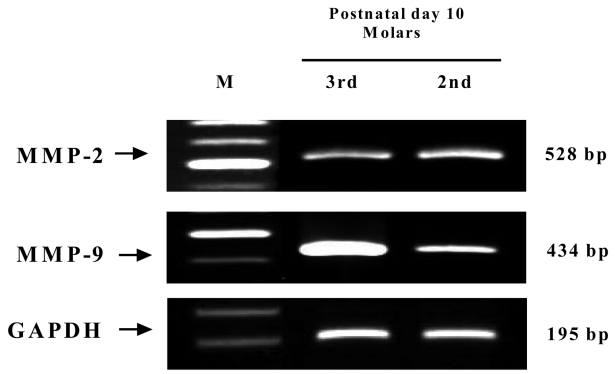

Fig. 2. Amplicons of MMP-2 and MMP-9 genes were generated in both upper 2nd and 3rd molars. MMP-2 expression level was higher in the 2nd molars than in the 3rd molars, whereas MMP-9 expression level was higher in the 3rd molars. Molecular marker (M) was 100 bp ladder.

The mRNA expressions of MMP-2 and MMP-9 from the upper 2nd molars at postnatal days 4 (early bell stage), 7 (crown stage) and 10 (root stage) were also analyzed to elucidate their roles in tooth development. They were all up-regulated at the crown and root stages (Fig. 3).

Analysis of MMP protein expression

Western blot analysis was performed using antibodies against MMP-2 and MMP-9 proteins that are represented as approximately 60 and 90/120 kDa in size, respectively and MMP-9 was shown as 2 isoforms. MMP-2 expression was stronger in the 2nd molars than that in the 3rd molars, as in

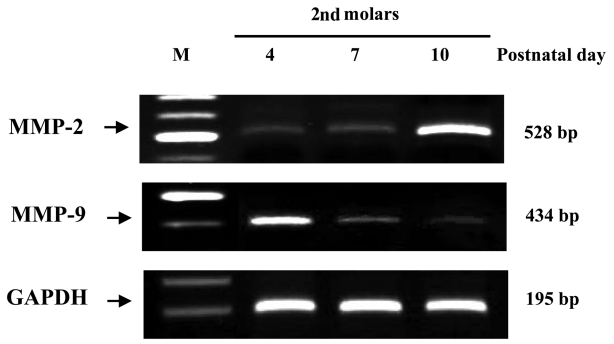

Fig. 3. Expressions of MMP-2 and MMP-9 genes from the 2nd molar germs at the early bell, crown and root stages. mRNA expression of MMP-2 was much increased at the root stage, whereas mRNA expression level of MMP-9 was higher at the cap stage. Molecular marker (M) was 100 bp ladder.

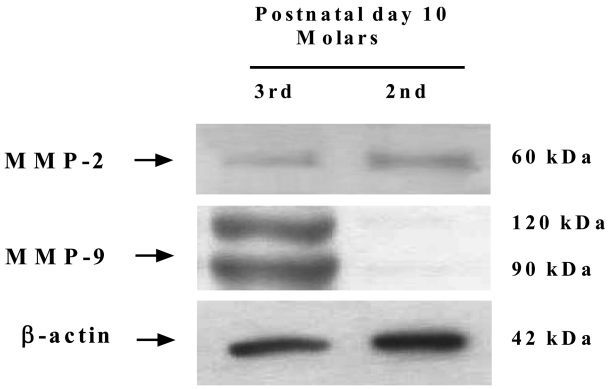

Fig. 4. Western blot for MMP-2 and MMP-9 expression in the cap-staged 3rd and root-staged 2nd molars. The expression level of MMP-2 was higher in the 2nd molars, whereas that of MMP-9, represented as 2 spliced forms, was higher in the 3rd molars.

mRNA expression. MMP-9 expression was represented as 2 isoforms which were approximately 120 kDa and 90 kDa in size. In contrast to MMP-2 expression, MMP-9 expression was stronger in the 3rd molars than that in the 2nd molars, as in mRNA expression (Fig. 4). These results were similar to those of mRNA expression. To investigate in more detail expression of MMP-2 and -9 during tooth development, protein levels of the 2nd molars were determined at postnatal days 4, 7 and 10. The expression of MMP-2 increased from the early bell to the root stages in a time dependent manner. However, MMP-9 was expressed only in the early bell stage of the 3rd molars (Fig. 5).

Immunofluorescent Findings

The negative control omitting the primary antibodies were all negative in reaction.

MMP-2: At postnatal day 10, tall columnar preameloblasts or ameloblasts at the root-staged 2nd molar exhibited strong, but transient reactivity against MMP-2 (Fig. 6a). Notably, strong immunoreactivities were seen at the basement membrane which temporally separated ameloblasts/preameloblasts from odontoblasts/preodontoblasts (Fig. 6b). However, its expression in odontoblasts was weak and different tooth-forming undifferentiated cells at the cap-staged germ did not show any immunoreactivity to MMP-2 (Fig. 6a).

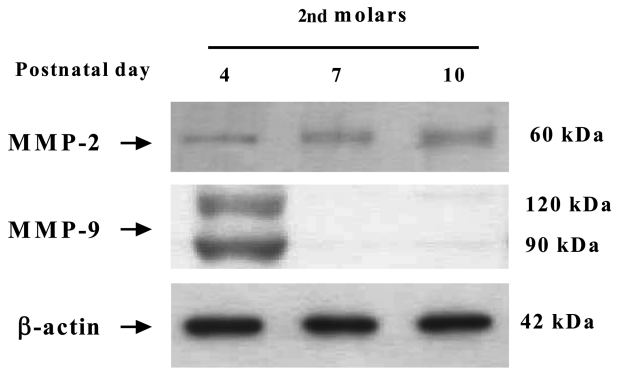

Fig. 5. Proteins levels in the 2nd molars at postnatal days 4, 7 and 10, which were at the early bell, the crown and the root stages, were determined. The expressions of MMP-2 increased in a time dependent manner. In contrast, the expression of MMP-9 was seen at the early bell stage.

MMP-9: At postnatal day 10, tall columnar odontoblasts at the root-staged 2nd molar exhibited strong immunoreactivities to MMP-9. Reactivity was also strongly expressed in ameloblasts, but transient (Fig. 6c). Immunoreactivities at the cap-staged 3rd molars were also seen, but strong reactivity was seen at the follicular tissues surrounding the germ (Fig. 6d).

Discussion

During tooth development, dynamic morphological changes occur in tooth germs. These changes include the degradation of basement membrane between preameloblasts and odontoblasts (Giannelli *et al*., 1997; Koshikawa *et al*., 2000; Goldberg *et al*., 2003), degradation of matrix proteins in the enamel and the dentin for mineralization and crystallization (Robinson *et al*., 1998; Woessner, 1998; Fanchon *et al*., 2004) and resorption of perifollicular tissues for tooth movement and growth (Bartlett *et al*., 2003). It has been well known that MMPs, as a family of proteolytic enzymes, have been implicated in degrading extracellular matrices during tissue morphogenesis and organogenesis (Sternlicht and Werb, 2001; Bourd-Boittin *et al*., 2005).

MMP-2 (gelatinase A) and MMP-9 (gelatinase B) have been suggested to degrade collagen IV in basement membrane and be activated by membrane type-1 matrix metalloproteinase (MT1-MMP) (Sato *et al*., 1994). The expression of MMP proteins from preodontoblasts and preameloblasts in the present study implied that degradation of basement membrane was a prerequisite for the differentiation of both cells, as well as mineralization.

In the present study, both mRNA and proteins of MMP-2

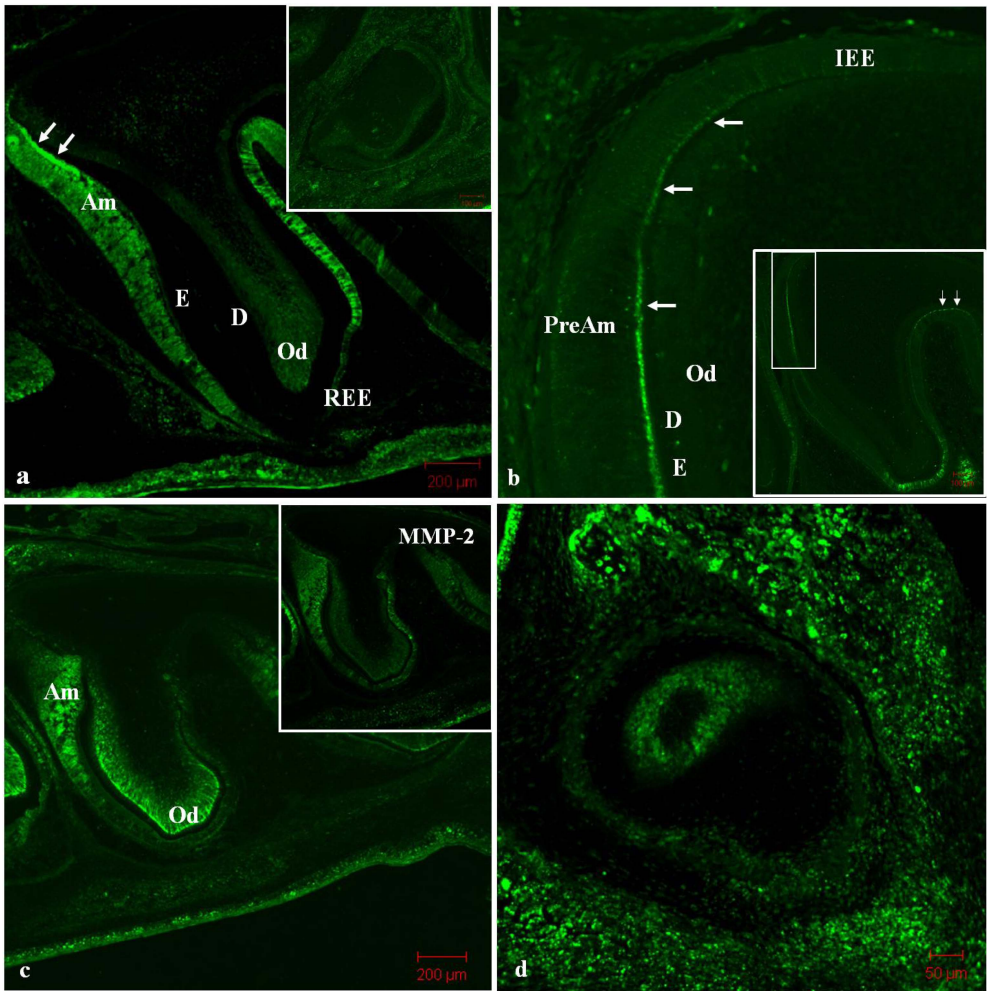

Fig. 6. Immunofluorescent findings of MMP-2 and -9 in developing rat tooth germs. (a) Strong immunoreactivities against MMP-2 are seen in tall columnar preameloblasts or ameloblasts at the root-staged 2nd molar at postnatal day 10. However, immunoreactivities in odontoblasts were weak. Strong immunoreactivities (arrows) are also seen in the basement membrane between preameloblasts and preodontoblasts. (Insert) All kinds of tooth-forming undifferentiated cells at the cap-staged 3rd molar germs at the same day did not show any immunoreactivities to MMP-2. (b) (Insert) Immunoreactivities against MMP-2 were transient, but seen at the cuspal region. Reactivities were also seen at the basement region (small arrows). Rectangular box in the insert was magnified. Strong immunoreactivities against MMP-2 (larger arrows) were seen at the basement membrane which lies between ameloblasts/preameloblasts and odontoblasts/preodontoblasts. (c) Strong immunoreactivities to MMP-9 are seen in tall columnar odontoblasts at the root-staged 2nd molar germ at postnatal day 10. Immunoreactive ameloblasts are also seen. (Insert) Adjacent section to the section for MMP-9 in (c) shows that MMP-2 is mainly expressed in ameloblasts, rather than odontoblasts. (d) Immunoreactivities to MMP-9 at the cap-staged 3rd molar germ were weak; however, strong reactivities were seen at the follicular tissues surrounding the germ. Am: ameloblasts, PreAm: preameloblasts, E: enamel, D: dentin, Od: odontoblasts, REE: reduced enamel epithelium, IEE: inner enamel epithelium.

expression was higher in the 2nd molars than that in the 3rd molars. At postnatal day 10, tall columnar ameloblasts at the root-staged 2nd molar exhibited strong reactivity against MMP-2. However, its expression in odontoblasts was weak and different tooth-forming undifferentiated cells at the cap-staged germ did not show any immunoreactivity to MMP-2. These results suggested that MMP-2 may be involved in the enamel matrix degradation. Notably, strong immunoreactivities were also seen at preameloblasts and the basement membrane which separates them from preodontoblasts, suggesting that MMP-2 might also be implicated in degradation of the basement membrane. Involvement of MMP-2 in degradation of the membrane during tooth development is also supported by several reports showing that MMP-2 cleaved laminin-5, which is a component of basement membrane, inducing epithelial mobility for tooth development (Giannelli *et al*., 1997; Koshikawa *et al*., 2000).

In the present study, in contrast to MMP-2, expression levels of both MMP-9 mRNA and proteins were unexpectedly higher in the 3rd molars than in the 2nd molars, as in mRNA expression. MMP-9 protein was detected in both ameloblasts and odontoblasts, suggesting that they may be involved in mineralization of dental hard tissue. MMP-9 expression in the 3rd molars was unexpectedly weak from immunofluorescent detection. This result was not concurrent with the western blot and RT-PCR results. However, strong

immunoreactivities were detected in perifollicular tissues. Thus, higher expression of MMP-9 in the 3rd molars was considered to be resulting from perifollicular tissues instead of tooth germs themselves, which were collected from the sampling.

All together, the present study demonstrated temporal and spatial expression patterns of MMP-2 and -9 mRNAs, as well as proteins, during tooth development. Both proteins may influence the events of tooth development. Specifically, MMP-2 may be involved in degradation of basement membrane, as well as degradation of dental hard tissue substance, during maturation. MMP activities are controlled by both transcriptional and translational regulations, latent proform activation and inhibitory action of tissue inhibitors of metalloproteinases (Smith *et al*., 2002; Yoshiba *et al*., 2003, 2007). Since the present study investigated only transcriptional and translational regulations of MMP-2 and -9, further studies are needed to elucidate other pathways for MMP activity control during tooth development.

Acknowledgements

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