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Expression of Tissue Inhibitors of Metalloproteinases in Developing Rat Tooth Germs

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Abstract : Tissue inhibitors of metalloproteinases (TIMPs) are a family of secreted molecules that were identified as natural inhibitors of matrix metalloproteinases (MMPs). Tooth histomorphogenesis and cytodifferentiation are accompanied by rapid changes in cellular organization and remodeling of the extracellular matrix, in which MMPs and TIMPs might be expected to play significant roles. This study examined the expression and localization of TIMP-1 and TIMP-2 during the molar development of rats.

The expression patterns of TIMPs were determined from Sprague-Dawley rat pups including the developing molars using RT-PCR, western blot and immunofluorescent staining.

Gene and protein quantification analyses showed that both TIMPs increased from the cap stage to the root stage tooth germs. In contrast, the immunofluorescent data showed that they were expressed slight differentially. TIMP-1 was strongly expressed in secretory ameloblasts and moderate immunoreactivity was observed along the basement membrane. TIMP-2 expression was also detected in the basement membrane. Although strong immunoreactivity was observed in the secretory ameloblasts and enamel matrix itself, differentiated odontoblasts showed weak reactivity. However, little reactivity for both TIMPs were detected in the cap stage tooth germs and surrounding tissues.

These distinct temporospatial expression patterns of TIMPs suggest that the TIMPs may play a variety of roles including dental hard tissue formation during molar tooth development.

Keywords : MMP, TIMP, Tooth development

Introduction

Tissue inhibitors of metalloproteinases (TIMPs), which exist in most tissues, are the endogenous inhibitors of matrix metalloproteinases (MMPs), one of the proteases that can break down the entire extracellular matrix (Chang and Werb 2001, Baker et al. 2002). They form a non-covalent 1 : 1 stoichiometric complex that can resist heat denaturation and proteolytic degradation, and perform important roles in remodeling of the extracellular matrix by inhibiting the proteolytic activities of MMPs (Gomez et al. 1997). In the pericellular environment, the balance between MMPs and TIMPs determines not only the events, such as wound healing and tissue remodeling, but pathological conditions, such as tumorigenesis or metastasis (Lambert et al. 2004). Thus far, 4 types of TIMPs have been characterized and named TIMP-1, -2, -3 and -4, respectively (Woessner and Nagase 2000). Although every TIMP represses the MMPs proteolytic activity, they differ from each other in many respects including solubility, interaction with proMMP, and regulation of expression (Greene et al. 1996). Recent studies of TIMP also provide evidence that can prove the ability of the multifunctional activities for cellular proliferation, apoptosis and differentiation, apart from the inhibition activity for MMPs (Baker et al. 2002, Lovelock et al.

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2005).

Throughout their process of development, teeth that are generated by the reciprocal induction of the epithelium and ectomesenchyme show a series of complex morphological changes (Jernvall and Thesleff 2000, Miletich and Sharpe 2003). In general, these are classified as the bud, cap, and bell stages depending on the shapes of the enamel organs formed from the dental epithelium. At the end of the bell stage, ameloblasts that are differentiated from the inner dental epithelium appear in the crown staged tooth germ, with odontoblasts differentiated from dental papilla showing the formation of dental matrix. Root formation follows the future cementoenamel junction with the formation of Hertwig's epithelial rooth sheath, consisting of two layers of an epithelial band, and the epithelial diaphragm can be observed at this stage. Along with these changes in cellular organization, the process of tooth development, including breakdown of the basement membrane that exists between preameloblasts and odontoblasts (Giannelli et al. 1997, Goldberg et al. 2003) and remodeling of the extracellular matrix, such as the resorption of perifollicular tissue for tooth movement and growth (Bartlett et al. 2003), can be a excellent model for examining the role of MMPs and TIMPs during the process of organ formation. Although some recent studies presented the distribution of MMPs and TIMPs during tooth development using *in situ* hybridization and immunohistochemistry, all were concerned only with the early stages of tooth development, and no studies on the expression after the formation of the hard tissue, such as dentin and enamel, and the quantitative changes utilizing molecular biological techniques have been performed (Yoshiba et al. 2003, 2006).

Therefore, this study examined the temporospatial distribution pattern by immunofluorescence using the TIMP-1 and 2 antibodies in the process of rat molar development and analyzed the mRNA and protein expression of TIMP-1 and 2 by RT-PCR and Western blotting to examine the role of TIMPs during tooth development.

Materials and Methods

1. Removal of tooth germs

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

2. Preparation for tissue sections and general histology

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

3. RT-PCR for TIMP-1 and TIMP-2 mRNA expression

The extracted tooth germs were frozen immediately in liquid nitrogen for total RNA extraction. The extraction was performed using a Trizol® Reagent (Gibco BRL, MD, USA). Before performing PCR, any DNA contamination was removed by treating the extracted DNA with DNase I (Gibco BRL, MD, USA). The TIMP-1 primer sequences were 5′ AAC AGT GTT CAG GCT TCA GCT TT 3′ for the forward and 5′ GTG TGC ACA GTG TTT CCC TGT T 3′ for the reverse, generating an expected PCR product of 218 bp. The TIMP-2 primer sequences were 5′ GCA GAT AAA GAT GTT CAA AG 3′ for the forward and 5′ TAA AGT CAC AGA GGG TAA TG 3′ for the reverse, generating an expected PCR product of 167 bp. The housekeeping gene, GAPDH was amplified using the primers with sequences of 5′ CCA TGG AGA AGG CTG GGG 3′ for the forward and 5′ CAA AGT TGT CAT GGA TGA CC 3′ for the reverse, generating an expected product of 195 bp. The RNA samples were quantified using a UV spectrophotometer and qualified by obtaining the OD 260/280 ratios >1.8. AccPower® RT PreMix (Bioneer, Daejeon, Korea) was used for reverse transcription. For PCR, mixtures of the total RNA and Oligo dT_{18} were added to an AccPower RT PreMix tube (Bioneer, Daejeon, Korea). cDNA synthesis was performed by incubating these mixtures at 42°C for 60 min. For PCR, AccPower® PCR PreMix (Bioneer, Daejeon, Korea) was used. Briefly, after adding the cDNA and primers to AccPower® PCR PreMix, PCR was per-

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formed in a Perkin-Elmer GeneAmp PCR system 2400 (Applied Biosystems/Perkin Elmer, Foster City, CA, USA) with the following profile: denaturation for 30 sec at 95°C, annealing for 30 sec at 57°C and 30 sec extension step at 72°C. Preliminary experiments were performed to determine the optimum number of PCR cycles. The products were resolved on a 1% agarose gel and visualized with ethidium bromide. The product size was confirmed using 100 bp (Takara, Otsu, Shiga, Japan). The DNA template was omitted for the negative control in PCR.

4. Western blot for TIMP-1 and TIMP-2 protein expression

A ready prep protein extraction kit® (Bio-RAD, Hercules, CA USA) was used to extract the protein from the tooth germs. Briefly, the extracted molar germs were resuspended and incubated in the protein extraction solution with a proteinase inhibitor cocktail (Roche, Mannheim, Germany) for 1 hr. The concentration of protein lysates was measured using Amersham GeneQuant Pro® (Amersham-Pharmacia Biotech, Arlington Heights, IL, USA). The lysates were boiled for 10 min in 3x SDS sample buffer, loaded onto 10% continuous gradient SDS-polyacrylamide gel and transferred to a Protran nitrocellulose membrane (Whatman GmbH, Dassel, Germany). The membrane was blocked with a 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 incubated with the purified rabbit polyclonal antibodies for TIMP-1 and TIMP-2 (Santa Cruz Biotech, Delaware, CA, USA) and the purified mouse monoclonal antibody for β-actin (Sigma-Aldrich Co., ST Louis, MO, USA) overnight at 4°C with gentle shaking. After washing with TBS-T 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. The bound antibodies to TIMP-1 and TIMP-2 were visualized with Lumiglo reagent (Cell signalling, Beverly, MA, USA).

5. Immunofluorescent staining

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

Results

1. Histological findings

The postnatal day 10 maxilla contained the 2nd molar showing the morphology of a root stage tooth germ and the 3rd molar tooth germ with the morphology of the cap stage. The 2nd molar tooth germ consisted of the following: cuboidal shaped odontoblasts to generate dentin matrix, which were differentiated from the dental papilla, long columnar ameloblasts being able to secrete enamel matrix, which are derived from inner enamel epithelium, pulp cells and reduced dental epithelium. The 2nd molar showed epi-

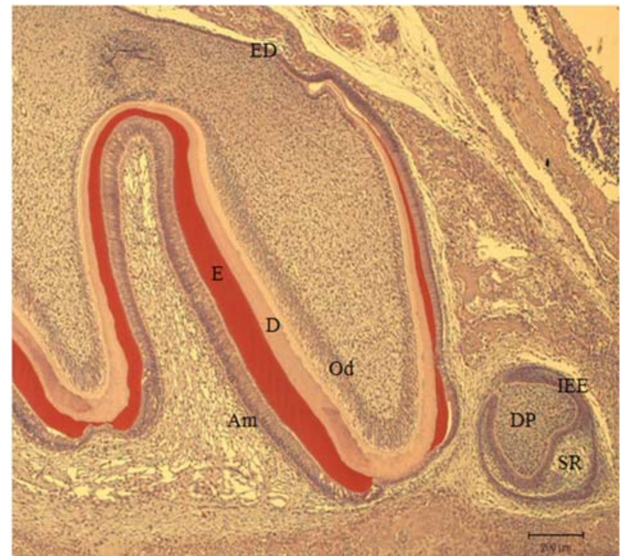

Fig. 1. The cap stage 3rd molar and root stage 2nd molar at postnatal day 10. The root stage germ shows the epithelial diaphragm (ED), enamel (E)-forming ameloblasts (Am) and dentin-forming odontoblasts (Od), whereas the cap-stage germ shows dental papilla (DP), inner enamel epithelium (IEE) and stellate reticulum (SR). ×60, H-E stain.

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thelial diaphragm at the cervical region of the crown with Hertwig's epithelial root sheath formation. The 3rd molar tooth germ had the structure of the enamel organ composed of inner enamel epithelium, outer enamel epithelium, stellate reticulum and dental papilla, with dental follicles enclosing (Fig. 1).

2. Analysis of TIMPs mRNA expression

RT-PCR was used to compare the expression of TIMP-1 and -2 mRNA depending on its stage during rat molar tooth development. The amount of TIMP-1 mRNA in the maxillary 2nd molar tooth germ at postnatal day 4, 7 and 10 increased in a time dependent manner. Compared to the in the cap stage 3rd molar germ at postnatal day 10, the root stage 2nd molar showed higher levels of expression (Fig. 2). A similar pattern of changes was found in the RT-PCR for TIMP-2 (Fig. 3).

3. Analysis of TIMPs protein expression

Western blot using rabbit polyclonal IgG TIMP-1 and -2 antibodies was performed to identify the changes in TIMP-1 and -2 protein expression. Little TIMP-1 protein was observed on day 4 and protein expression was started on day 7, showing an increased level on day 10, according to

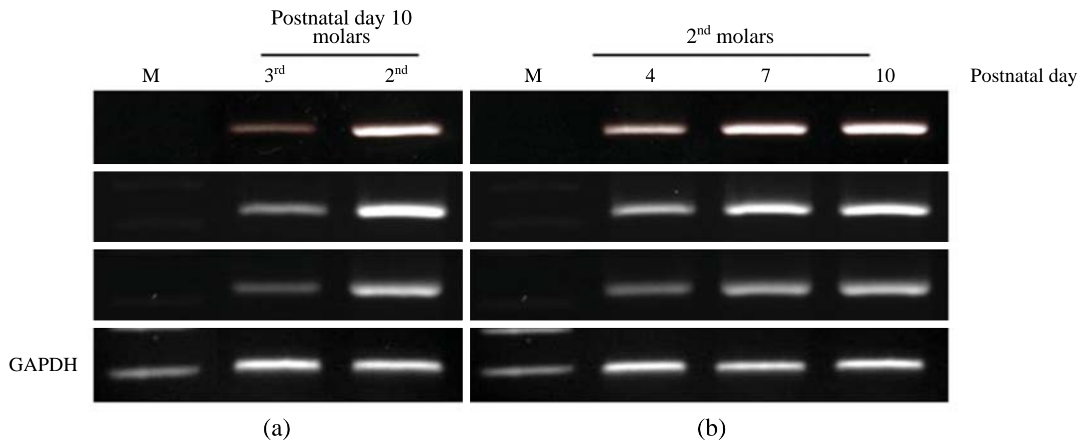

Fig. 2. (a) Expression of the TIMP-1 genes in the upper 2nd and 3rd molars at postnatal day 10. The level of TIMP-1 expression is higher in the 2nd molars than in the 3rd molars. (b) Expression of TIMP-1 from the 2nd tooth germs at the bell, crown and root stages. mRNA expression of TIMP-1 much increased at the crown and root stages. Molecular marker (M) is 100 bp ladder. The results were confirmed by three repeated tests.

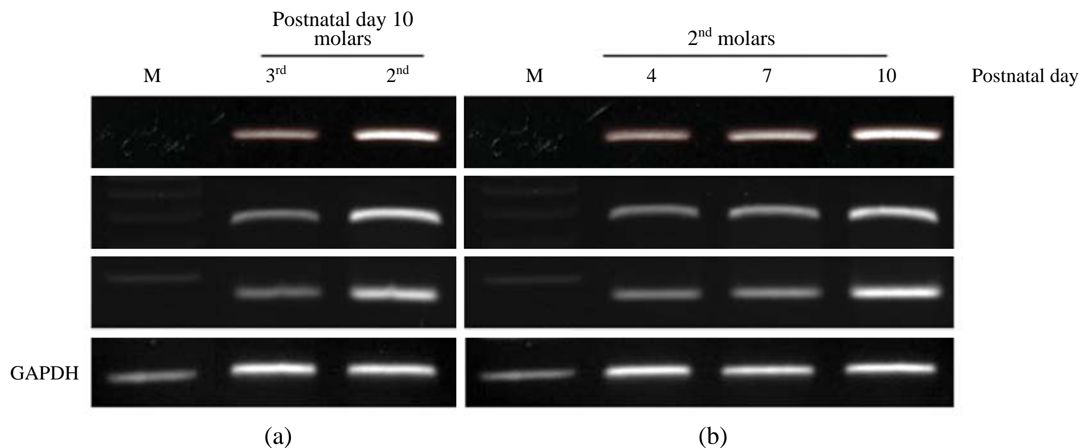

Fig. 3. (a) Expressions of TIMP-2 genes in the upper 2nd and 3rd molars at postnatal day 10. The TIMP-2 expression level was higher in the 2nd molars than in the 3rd molars. (b) Expressions of TIMP-2 from the 2nd tooth germs at the bell, crown and root stages. mRNA expression of TIMP-2 increased considerably at the root stage. Molecular marker (M) is 100 bp ladder.

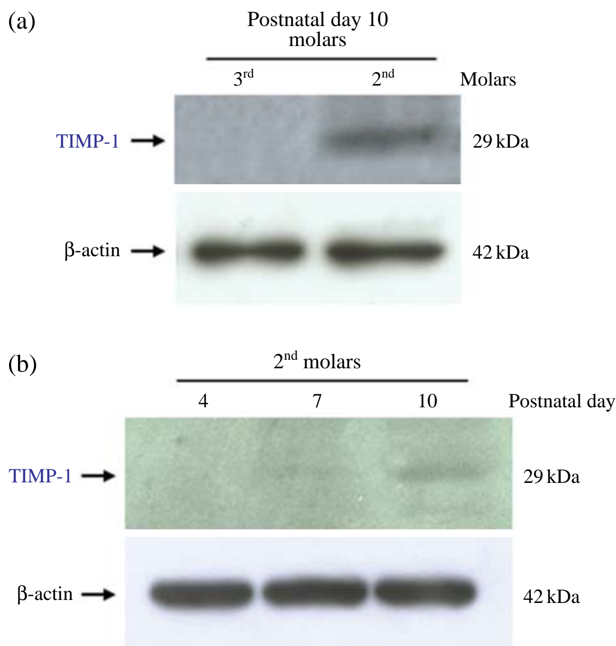

Fig. 4. (a) Western blot for TIMP-1 expression in the cap stage 3rd and root stage 2nd molars. The level of TIMP-1 expression was higher in the 2nd molars. (b) Expressions of TIMP-1 protein from the 2nd tooth germs at the bell, crown and root stages. Protein expression of TIMP-1 much increased at the root stage.

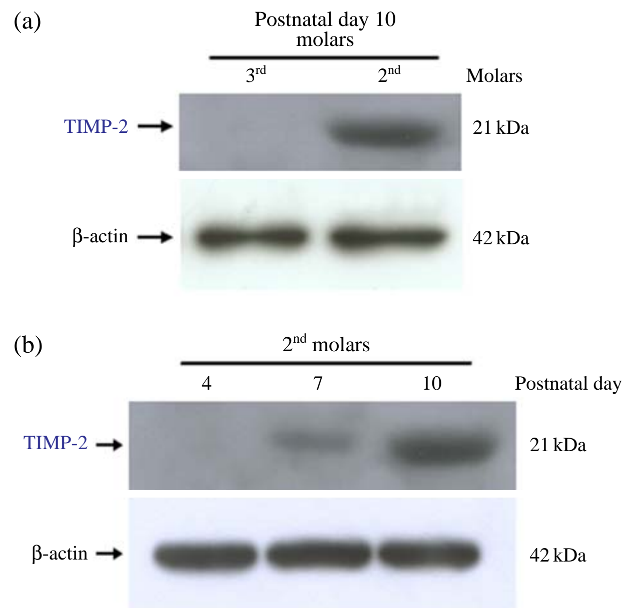

Fig. 5. (a) Western blot for TIMP-2 expression in the cap stage 3rd and root stage 2nd molars. The expression level of TIMP-2 was higher in the 2nd molars. (b) Expressions of TIMP-2 protein from the 2nd tooth germs at the bell, crown and root stages. Protein expression of TIMP-2 much increased at the root stage.

western blot analysis on the postnatal day 4, 7 and 10 maxillary 2nd molar tooth germ. Although little TIMP-1 protein was observed in the postnatal day 10 3rd molar germ, intense expression was noted in the 2nd molar tooth germ at the same stage. TIMP-2 protein expression was similar to that of TIMP-1 (Figs. 4a, 5a). To examine the expression of TIMP-1 and -2 in more detail during tooth development, the protein levels of the 2nd molars at postnatal days 4, 7 and 10 were determined. The expressions of both TIMPs increased from the bell stage to the root stages in a time dependent manner (Figs. 4b, 5b).

4. Immunofluorescent findings

Immunofluorescent staining was carried out to reveal the TIMP-1 and 2 protein distribution depending on the stage during rat molar tooth development. From the maxillary 2nd molar tooth germ showing the morphology of the bell stage at postnatal day 4, anti-TIMP-1 immunoreactivity was observed at the basement membrane between the differentiating ameloblasts and odontoblasts (Fig. 6a). On the other hand, positive responses at the basement membrane revealed transient reactions, which faded away as the dentin and enamel matrices formed. Differentiated ameloblasts stained intensively at postnatal day 10 (Fig. 6c). TIMP-2 immunoreactivity was also observed in the basement membrane in the bell stage 2nd molar tooth germ (Fig. 6d), which disappeared on postnatal day 10. Moreover, the secreting ameloblasts and enamel matrix itself of the maxillary 2nd molar tooth germ at postnatal day 10 showed distinct immunopositivity, and relatively weaker staining for odontoblasts (Fig. 6e). On the other hand, little TIMP-1 and -2 reactivity was detected in the maxillary 3rd molar tooth germ at postnatal day 10, which had the morphology of the cap stage (Fig. 6b, e).

Discussion

Remodeling of the extracellular matrix functions is an important modulator regulating the tooth development. Previous studies reported that TIMPs could play a significant role in tooth development (Sahlberg et al. 1999, Yoshiba et al. 2003, 2006). This study also presented similar results, showing a temporospatially regulated expression pattern of TIMP-1 and -2 during the developmental process in the maxillary 2nd molar and 3rd molar.

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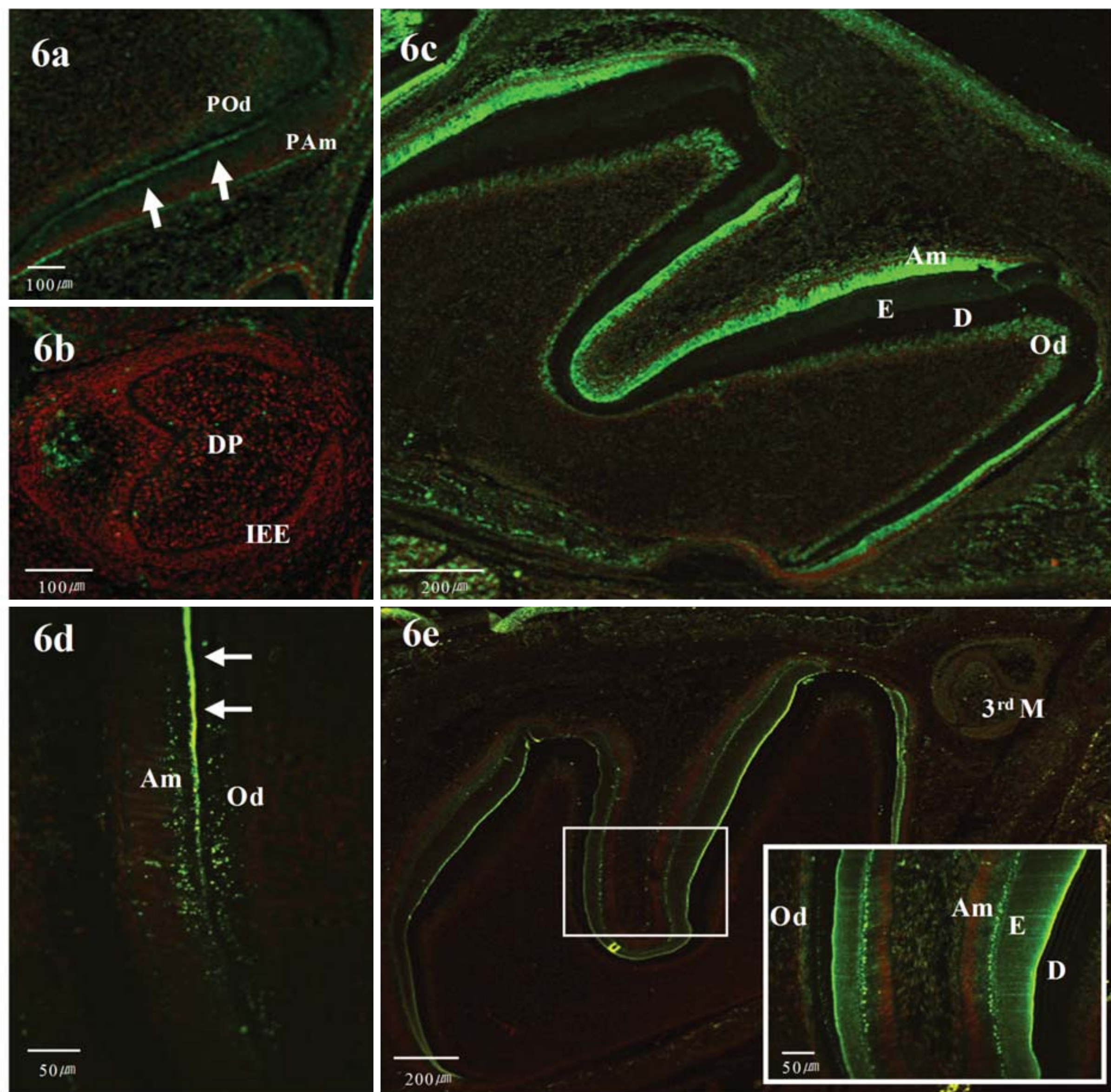

Fig. 6. Immunofluorescent findings of TIMPs in developing rat tooth germs. (a) TIMP-1 immunoreactivity (arrows) was observed in the basement membrane between preameloblasts (PAm) and preodontoblasts (POd) at the bell stage 2nd molar at postnatal day 4. (b) All kinds of tooth-forming undifferentiated cells at the cap stage 3rd molar germs at postnatal day 10 do not show any immunoreactivity against TIMP-1 anti-serum. DP: dental papilla, IEE: inner enamel epithelium. (c) Strong immunoreactivity against TIMP-1 anti-serum can be seen in tall columnar preameloblasts/ameloblasts at the root stage 2nd molar at postnatal day 10. The immunoreactivity for odontoblasts was relatively weak. Am: ameloblasts, E: enamel, D: dentin, Od: odontoblasts. (d) Strong immunoreactivity against TIMP-2 (arrows) can be seen at the basement membrane that lies between ameloblasts/preameloblasts and odontoblasts/preodontoblasts at the bell stage 2nd molar at postnatal day 4. Am: ameloblasts, Od: odontoblasts. (e) Strong immunoreactivity to TIMP-2 are seen in tall columnar ameloblast and enamel matrix at the root stage 2nd molar germ at postnatal day 10. The immunoreactivity for odontoblasts was relatively weak. Little reactivity was observed in the 3rd molar tooth germ (3rd M). Insert magnified rectangular box. Am: ameloblasts, E: enamel, D: dentin, Od: odontoblasts.

In the present study, the TIMP-1 mRNA and proteins in the root stage 2nd molar were expressed at higher levels than those in the cap staged 3rd molar. Strong immunopositivity was observed in ameloblasts on the postnatal day 10 2nd molar, whereas little response was observed from the 3rd molar tooth germ at the same stage from an immunofluorescence study. This suggests that TIMP-1 can be related to the differentiation and maturation of ameloblasts that

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are responsible for secreting enamel matrix. Furthermore, an immunopositive reaction was observed transiently from the basement membrane at the bell stage, providing concrete evidence that TIMP-1 contributed to maintaining the integrity of basement membrane before the secretion of the tooth substrates.

The results for TIMP-2 showed similar aspects to TIMP-1. Therefore, it was assumed that they might play a common role in tooth development. On the other hand, slight differences were obtained from the immunofluorescence results, showing strong positive responses not only in the ameloblasts from the root stage 2nd molar tooth germ but in the enamel matrix, and relatively weak reactivity for odontoblasts subjected to secrete the dentin matrix. The above results suggested that TIMP-2 plays a key role in enamel formation mainly by regulating enamel biomineralization. In addition, laminin-5, which is one of the main components of the basement membrane, functions as a critical survival factor of ameloblasts (Ryan et al. 1999) and is the target substrate of MMPs (Caron et al. 2001, Seiki 2002). Therefore, the TIMP-2 protein, which is also found at the basement membrane, contributes to enamel formation by improving the survival rate of ameloblasts, and has the ability to protect laminin-5 from the proteolytic activity of MMPs (Yoshiba et al. 2003). On the other hand, little is known about the mechanism how the TIMP-2 bind to the matrix. Therefore, it will be important to identify the specific receptor for TIMP-2 will be needed.

The TIMP family consists of four members, and the individual TIMPs differ in their ability to inhibit various MMPs. TIMP-1 potentially inhibits the activity of most MMPs except for MMP-2. TIMP-2 also inhibits the activity of most MMPs, with the exception of MMP-9 (Woessner and Nagase 2000). In the present study, mRNA and protein for both TIMPs increased from the cap stage tooth germs to the crown stage germs. Interestingly, previous reports showed that expression patterns of MMP-2 showed the same changes in TIMPs-1 and -2 expression, but the changes in MMP-9 expression was the reverse at both the transcriptional and translational levels (Kim 2007). TIMPs also have been shown to assume specific functions independently of their MMP inhibitory activity (Hayakawa et al. 1992, 1994).

Overall, this study demonstrated the temporospatial expression patterns of TIMP-1 and -2 mRNA as well as the proteins during tooth development. Both proteins can affect the events of tooth development. In particular, TIMP-1 and -2 might be involved in the process of maintaining the integrity of the basement membrane and the formation of dental hard tissue matrix. In addition, TIMPs may also play roles independent of inhibiting the MMP activities. Since the present study investigated only the transcriptional and translational regulations of TIMPs, further studies will be needed to determine the mechanisms of the control TIMP activities at the cellular level using molecular biological techniques.

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흰쥐 발생기 치배에서 TIMPs 발현에 관한 연구

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간추림 : TIMPs는 MMPs의 자연적인 억제제로 알려진 분비 물질 군이다. 치아 발생과정 중의 조직 및 형태분화 과정 동안 세포의 유기체화와 세포외 기질의 개조에 있어 빠른 변화가 수반되고 이에 MMPs와 TIMPs가 관여하리라 생각되고 있다. 본 연구는 쥐의 구치부 치아 발생 과정 중 TIMP-1과 TIMP-2의 발현 및 위치를 구명하기 위하여 시도되었다.

발생중인 구치를 포함하고 있는 흰쥐 새끼로부터 TIMPs의 발현 양상을 RT-PCR, western blot, 면역형광염색법 등의 방법을 사용하여 분석하였다.

TIMP-1과 TIMP-2 mRNA와 단백질 발현 모두 모자시기 치배에서 치근기 치배로 갈수록 증가하였다. 반면 면역형광염색 양상은 약간의 차이를 보였다. TIMP-1은 분비기 법랑질모세포에서 강하게 발현되었고 아울러 기저막에서도 양성반응을 보였다. TIMP-2 역시 기저막과 분비기 법랑질모세포에서 강한 염색성을 보였으나 상아모세포에서의 염색성은 약하였고, 상대적으로 분비된 법랑기질에서 강한 양성 반응을 보였다. 한편 모자시기 치배나 주위조직에서는 TIMP-1 및 TIMP-2 모두 음성을 나타내었다.

이와 같은 독특한 TIMPs의 시공간적인 발현 양상은 TIMPs가 구치 발생 과정 동안 치아 경조직 형성을 포함한 다양한 역할을 수행할 수 있음을 시사하였다.

찾아보기 낱말 : MMP, TIMP, 치아발생

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