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Feasibility of CD147 for an Eruption-Related Molecule during Rat Molar Development

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Abstract : Understanding the genetic control of tooth eruption is one of the major issues in tooth development. Thus far, it is known that eruption-related molecules are secreted from follicular cells surrounding the germs and are related mainly to osteoclast formation. This study examined the involvement of CD147 and its downstream molecules in the eruption of rat developing molars using immunohistochemistry, RT-PCR and histomorphometry.

CD147 was expressed differentially in the cap (3rd molar germs) and root formation (2nd molar germs) stages in tooth development. CD147 was localized immunohistochemically in the follicular cells and osteoclasts as well as in the ameloblasts and odontoblasts. The expression pattern of CD147 and mmps was investigated because CD147 is an mmp inducer. The expression of both mmp-2 and -9 increased at the root formation stage compared to that at the cap stage and increased in a stage dependent manner. However, the level of mmp-13 was not changed notably. The histomorphometrical study suggested that the number of osteoclasts that appeared occlusal to the molar germs for the resorption of alveolar bone increased significantly during development.

These results suggest that CD147 may play an important role in the formation of the eruption pathway along with the mmps.

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Key words : CD147, Tooth eruption, mmps

Introduction

The molecular mechanisms for tooth eruption are largely unknown (Verma et al. 2005). Thus far, osteoclastic bone resorption has been the main focus of studies on tooth eruption. Osteoclasts remove the alveolar bone that overlies the developing tooth germs to prepare an eruption pathway. In osteopetrotic animals, in which the osteoclastic activity is attenuated by the lack of colony-stimulating factor activity, tooth eruption can be recovered by adding osteoclast precursors (Niida et al. 1997, Wise et al. 2000). Osteoclasts demineralize bone by forming a sealed acidic microenvironment and exposing the organic matrix in the bone.

However, despite the importance of osteoclasts in tooth eruption, collagen remodeling is still needed to

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produce an eruption pathway. Insufficient remodeling in periodontal tissue can generate resistance in collagen fiber networks, resulting in an impediment or delay in occlusal movement of the tooth germ. Hence, the remodeling of soft tissue and osteoclastic bone resorption is essential for successful tooth eruption. CD147, which is also called extracellular matrix metalloproteinase inducer (EMMPRIN), basigin or neurothelin, is a member of the immunoglobulin superfamily class of cell surface adhesion molecules that are regulated developmentally. This protein is an integral membrane receptor that mediates the internalization of bound cyclophilin (Yurchenko et al. 2001, 2002, 2005). CD147 stimulates the production of multiple matrix metalloproteinase as the name suggests. It is also an essential component in the cyclophilin mediated-signalling cascade in the presence of heparan sulfate, a subtype of glycosaminoglycan as the primary binding site (De Ceuninck et al. 2003). Recombinant purified or soluble secreted CD147 glycoprotein stimulates fibroblasts to produce high levels of matrix metalloproteinases (mmps), such as mmp-1, mmp-2 and mmp-3 (Sun and Hemler 2001, Norgauer et al. 2002).

Bone destruction is accomplished in two stages. In the first stage, osteoclasts create a sealed acidic environment through the action of a proton pump, resulting in bone demineralization and subsequent exposure of the organic matrix. The exposed organic matrix is degraded by the action of the lysosomal enzymes released, such as acid phosphatase and cathepsin. Although cathepsin is predominant in osteoclasts, cathepsin-/- mice osteoclasts are capable of bone resorption (Okaji et al. 2003). This suggests that other enzymes play a role in bone resorption. Indeed, the exposed matrix, including collagen, can be degraded by other enzymes, such as matrix metalloproteinase (mmp) (Beertsen et al. 2002) and tissue inhibitor of metalloproteinase (TIMP) (Sahlberg et al. 1999). Therefore, bone resorption in tooth eruption is accomplished by other cells, such as follicular cells and osteoclasts. mmp-9 expression is increased in cathepsin-/- mice osteoclasts (Okaji et al. 2003). Indeed, mmps have been reported to be involved in tooth eruption. They are secreted from the periodontal tissues, including the periodontal ligament, pulp tissue and the gingiva (Palosaari et al. 2003). They are involved in tooth eruption (Beertsen et al. 2002, 2003, Bartlett et al. 2003) and tooth morphogenesis including root growth (Sahlberg et al. 1999).

A range of eruption-related molecules may be involved in tooth eruption. In this study, mmp-2, -9 and 13 and CD147, the upstream modulator of mmps were examined to determine their relationships with tooth eruption.

Materials and Methods

1. Tissue preparation for microscopy

Sprague-Dawley rats were raised in Laboratory Animal Care-Approved Facilities. The tissues containing developing maxillary molars at postnatal days 3, 6 and 9 were immersion-fixed overnight in a 4% paraformaldehyde solution and decalcified with ethylene diamine tetra-acetic acid (pH 7.4) for a period of several weeks. The tissues were then dehydrated in a graded series of ethyl alcohol and embedded in paraffin. Five-μm-thick midsagittal sections were produced for H-E staining and immunohistochemistry.

2. RT-PCR

After the gingivae and alveolar bone that overlay the tooth germs were removed carefully, the upper 2nd molar germs at postnatal days 3, 6 and 9 and the upper 3rd molar germs at postnatal day 9 were extracted from the tooth crypts together with the surrounding follicular tissues using sterile forceps. These germs were frozen immediately in liquid nitrogen for total RNA extraction

Table 1. Sequences of the oligonucleotide primers for RT-PCR

Gene	Sequence (5′ to 3′)	Size (bp)	Genbank No.
CD147	F GCT GGC CTT CAC GTT CCT GAG T R CGT TCC TCT GGC GCA CAT TCT	788	BC_059145.1
MMP-2	F GAG ATC TGC AAA CAG GAC AT R GGT TCT CCA GCT TCA GGT AA	528	NM_031054
MMP-9	F CGG TAT TGG AAG TTC TCG AAT CAC R CAC ACG CCA GAA GTA TTT GTC ATG	434	NM_031055
MMP-13	F TGA CTA TGC GTG GCT GGA A R AAG CTG AAA TCT TGC CTT GGA	355	NM_133530
GAPDH	F CCA TGG AGA AGG CTG GGG R CAA AGT TGT CAT GGA TGA CC	195	AF_106860

using a Trizol Reagent (Gibco BRL, MD, USA). The RNA samples were quantified using a UV spectrophotometer.

The primers of CD147, MMP-2, MMP-9, MMP-13 and GAPDH as a reference were custom-designed and summarized in Table 1. First strand cDNA synthesis was carried out using Superscript II (Gibco BRL, MD, USA). The first reactions, components that consisted of Oligo (dT)12-18 (Gibco BRL, MD, USA) and RNA, were incubated at 70˚C for 10 min. For the 2nd reactions, the first strand buffer, DTT, dNTP mix and RNase inhibitor (Gibco BRL, MD, USA) were mixed gently in the first reactions and incubated at 42˚C for 2 min, which was followed by the addition of Superscript II and subsequent incubation for 50 min at 42˚C. The RT controls were performed using the same RT reaction mix except for the substitution of DEPC-H_2O for Superscript II. The PCR cycles were performed in a GeneAmp PCR system 2400 (Perkin-Elmer, CA, USA) using the following profile: denaturation for 1 min at 95˚C, annealing for 1 min at 53˚C for CD147 primers, 52˚C for mmp-2, 58˚C for mmp-9 and -13 and 60˚C for GAPDH and 1 min extension step at 72˚C. The final cycle was followed by a final extension step of 10 min at 72˚C. The DNA template was omitted for the negative control. The products were resolved on 1.2% agarose gel and visualized using ethidium bromide. The size was confirmed using a 1 kb DNA ladder (Gibco BRL, MD, USA).

3. Immunohistochemistry

Immunohistochemical staining was performed using Vectastain Elite ABC Kit (Vector Laboratories, CA, USA). Purified goat monoclonal anti-CD147 (Santa Cruz biotechnology, Inc., USA) was used as the primary antibody. Normal horse serum was used as the negative control. The sections were incubated in 0.3% H_2O_2 in water for 30 min to block the endogenous peroxidase, followed by incubation in a blocking serum for 30 min to block the non-specific reactions. They were then reacted in the primary antibody at 4˚C overnight and incubated in the biotinylated secondary antibody for 2 hrs. Finally, the sections were incubated in an avidine-biotin peroxidase complex for 30 min and developed with AEC.

4. Histomorphometry

The midsagittal sections were used to count the number of osteoclasts located occlusal to the maxillary 2nd molar germs at postnatal days 3, 6 and 9. Osteoclasts were counted within the area made by two vertical lines tangential to the mesial and distal surfaces of the germs. A student's t-test was used to test the significance.

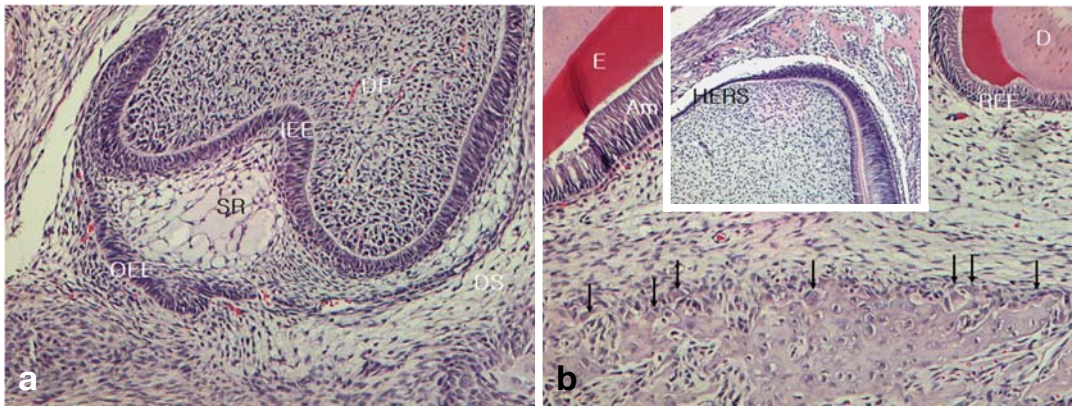

Fig. 1. (a) The upper 3rd molar germ at postnatal day 9. At the cap or early bell stage is the germ, which is composed of dental papilla (DP), dental sac (DS), stellate reticulum (SR), inner enamel epithelium (IEE) and outer enamel epithelium (OEE). ×100, H-E stain (b) The upper 2nd molar germ at postnatal day 9 is at the root formation stage, which is characterized by the presence of a Hertwig epithelial root sheath (HERS in the insert) and reduced enamel epithelium (REE). Many osteoclasts (arrows) can be seen on the surface of the alveolar bone. Am: ameloblasts, D: dentin, E: enamel, ×250, H-E stain.

Results

1. Histological and histomorphometrical findings

The upper 3rd molar germs were at the cap or early bell stage in tooth development at postnatal day 9. These germs displayed the enamel organ, dental papilla and dental sac or dental follicle. The enamel organ was composed of the inner and outer enamel epithelia separated by stellate reticulum (Fig. 1a). In contrast, the upper 2nd molar germs at the same day were at the root formation stage, when the eruptive movement of the germs began. The formation of the enamel was completed and the epithelial diaphragm was observed to determine the extent of root formation (Fig. 1b). The upper 2nd molar germs were at the early bell and crown stages at postnatal days 3 and 6, respectively.

The upper 2nd molar germs were confined within the connective tissue of the dental sac and bony crypts. Blood vessels and many monocytes were observed within the bony crypt. Multinucleated cells were found mainly on the surface of the developing alveolar bone. There was a significantly higher number of osteoclasts that appeared occlusal to the developing tooth germs at the root formation stage (11.8 ± 2.1) than at the cap stage (6.7 ± 1.7) ($p < 0.05$).

2. Immunohistochemical localization of CD147

1) 3rd molar germs

The inner enamel epithelia and papilla cells showed weak immunoreactivity against CD147 at postnatal day 9. However, the follicular cells adjacent to the germs exhibited moderate immunoreactivity (Fig. 2a).

2) 2nd molar germs

At postnatal day 3, immunoreactivity was observed in the follicular cells surrounding the developing tooth germs. However, the reactivity was weak in the inner enamel epithelium and dental papilla cells (Fig. 2b). At postnatal day 6, strong reactivity was observed in both enamel-forming ameloblasts of the long columnar shape at the secretory stage and dentin-forming odontoblasts (Fig. 2c). Reactivity was also observed in the

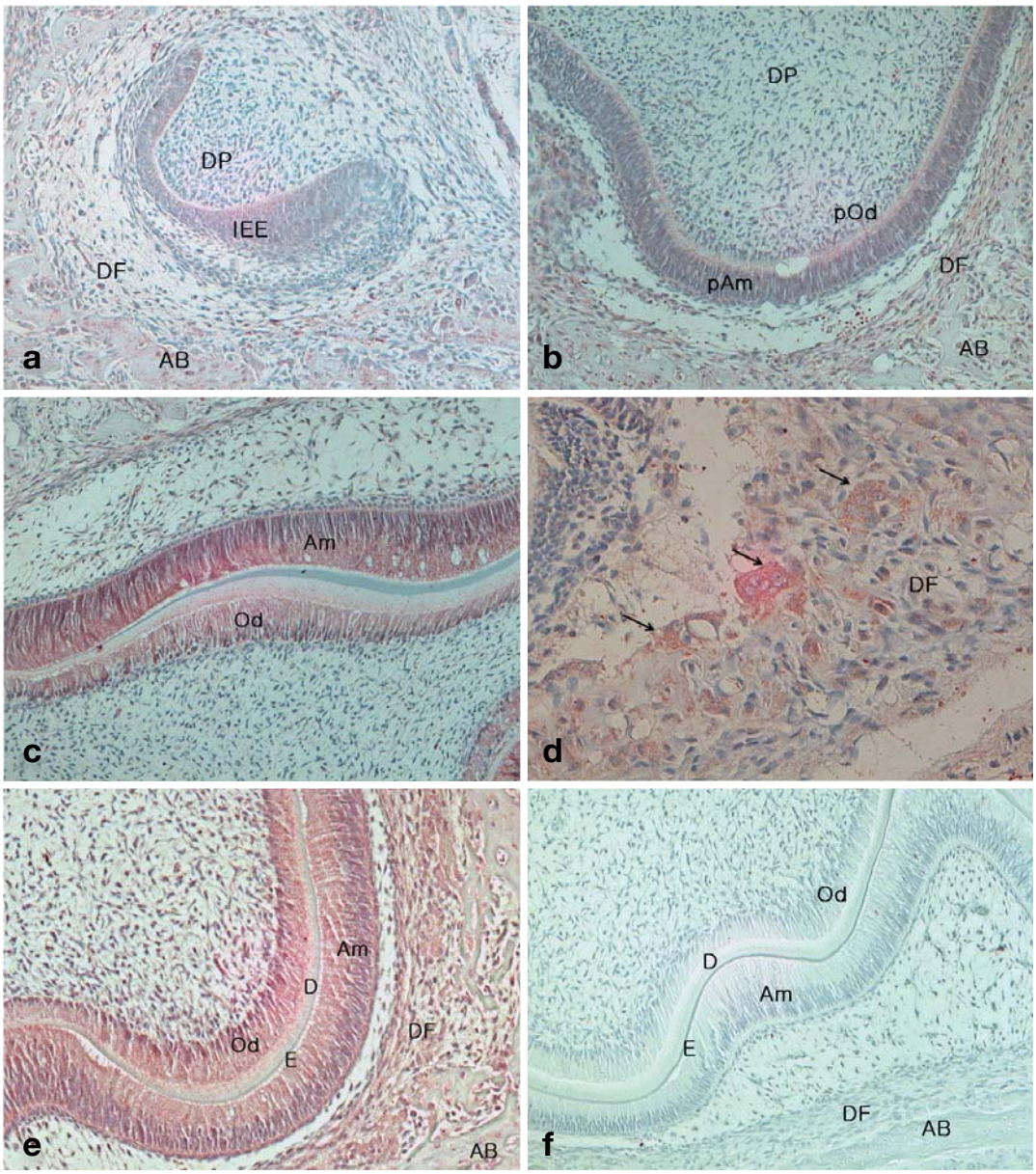

Fig. 2. (a) The dental epithelium and papilla cells of the 3rd molar tooth germ at the cap stage at postnatal day 9 show weak immunoreactivity to anti-CD147 sera. ×100 (b) The 2nd molar germ of the early bell stage at postnatal day 3 shows very weak reactivity in follicular tissues as well as the inner enamel epithelium and dental papilla cells. ×100 (c) The 2nd molar tooth germ of the crown stage at postnatal day 6 shows strong immunoreactivity to anti-CD147 sera in both ameloblasts at the secretory stage and dentin-forming odontoblasts. ×250 (d) Strong reactivity can be seen in the follicular cells and osteoclasts (arrows) in the 2nd molar tooth germ at postnatal day 6. ×250 (e) Strong reactivity is demonstrated in not only ameloblasts and odontoblasts but also follicular cells in the 2nd molar tooth germ of the root stage at postnatal day 9. ×250 (f) No reactivity was observed in the negative control. ×250. AB: alveolar bone, Am: ameloblasts, D: dentin, DF: dental follicle, DP: dental papilla, E: enamel, IEE: inner enamel epithelium, pAm: preameloblast, pOd: preodontoblast.

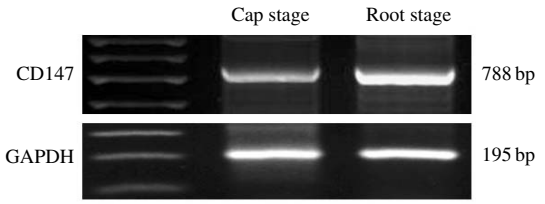

Fig. 3. Amplicons of 788 bp CD147 mRNA were generated from both the upper 2nd and the 3rd molar germs by RT-PCR, but the expression level was much higher at the root formation stage. The molecular size marker is a 1 kb ladder.

follicular cells and osteoclasts. However, the Hertwig epithelial root sheath did not show any immunoreactivity (Fig. 2d). At postnatal day 9, strong immunoreactivity continued in follicular tissues as well as in the ameloblasts and odontoblasts (Fig. 2e). No reactivity was observed in the negative control (Fig. 2f).

3. CD147 mRNA expression during tooth development

The levels of CD147 mRNA expression in the cap (the upper 3rd molar germs) and root formation stages for eruption (the upper 2nd molar germs) at postnatal day 9 were compared. Amplicons of the expected size (788 bp) were generated from both molar germs. However, the expression level at the root formation stage was much higher than that at the cap stage (Fig. 3).

4. mRNA expression of mmps during tooth development

RT-PCR was performed to elucidate changes in the expressions of mmp-2, -9 and -13 mRNAs during development of the 2nd and 3rd molar germs. The expression of mmp-2 and -9 were much higher at the root formation stage than that at the cap stage (Fig. 4a). Also, the expressions in the 2nd molars increased from the late bell to the root stage in a time dependent manner (Fig. 4b). To the contrary, the level of mmp-13 mRNA was not changed according to the developmental stages

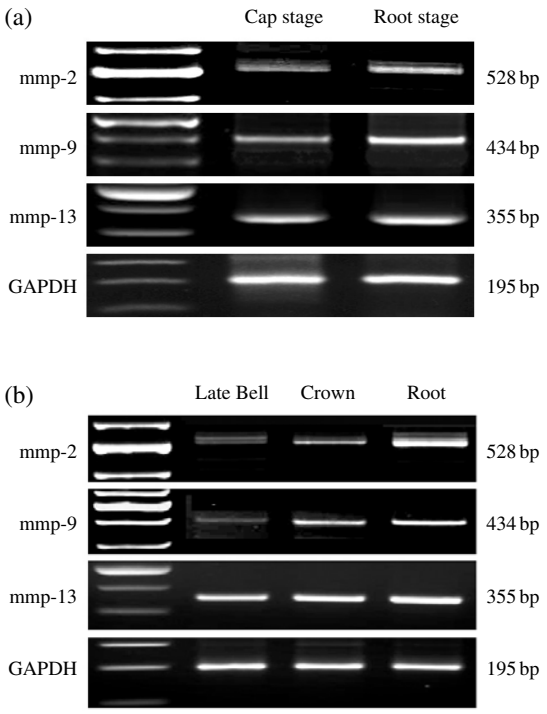

Fig. 4. (a) Expressions of mmp-2 and -9 mRNA between the cap stage (3rd molar germs) and the root formation stage (2nd molar germs) were compared at the transcription level. The expression levels of all the mmps were higher at the root formation stage. However, the mmp-13 mRNA level was not different significantly. (b) Expression levels of mmp-2 and -9 mRNA were compared in the 2nd molar germs. The levels increased from the late bell to the root formation stage. However, the mmp-13 mRNA level was not changed stage-dependently. Molecular marker is 1 kb ladder.

of the tooth germs (Fig. 4a, b).

Discussion

The eruptive movement of developing tooth germs begins soon after the formation of the crown outline followed by root formation. At postnatal day 9, the upper 2nd molar germs were at the root formation stage when they began eruptive movement, whereas the 3rd molar germs were at the cap stage before movement.

During the formation of an eruption pathway, bony crypts undergo morphological changes. One is the resorption of alveolar bone, which is mediated by osteoclasts. In this study, osteoclasts located occlusally were found to be involved in the formation of an eruption pathway. There were a larger number of osteoclasts at the root formation stage than in the cap stage. This increased number of osteoclasts at the root formation stage may imply that tooth eruption accelerates as the root grows, giving an impetus to eruption.

Under physiological conditions, matrix collagen fibers in the bone are degraded partially by the phagocytic activity of the cells, contributing to the remodelling of the hard tissue-soft tissue interface. Although degraded collagen fibers were taken up by phagosomes and subsequently digested by fusion with lysosomes (Everts et al. 1996), the mmps also play a key role in the degradative processes of collagen (Beertsen et al. 2002, Holmbeck et al. 2005). Collagen fibrils are digested by these enzymes before being phagocytosed in the cells (Beertsen et al. 2002). However, for all their importance in bone resorption, there are few reports on the roles of mmps in tooth eruption.

mmps are biochemically distinct depending on their activity on collagen fibrils (Martel-Pelletier and Pelletier 1996). mmp-2 (gelatinase A) and -9 (gelatinase B) have a substrate preference to gelatin, which is denatured collagen. mmp-9 is expressed in osteoclasts (Blavier and Delaisse 1995), and contributes to the formation of bone marrow cavities and releasing VEGF from the matrix (Gerber et al. 1999). mmp-2 is secreted from connective tissue cells, including osteoblasts (Martignetti et al. 2001), and is essential for the degradation of collagen fibers (Creemers et al. 1998, Kerkvliet et al. 1999). MT1-mmp plays a role in collagen remodeling in molar eruption and root growth (Beertsen et al. 2002, 2003). In this study, the expression of mmp-2 and -9 were higher at the root formation stage than at the cap stage and increased in a stage-dependent manner. The high level of mmp-2 and -9 expression in the root formation stage implied that these enzymes may be closely involved in the resorption of collagen matrix during tooth eruption. However, the expression of mmp-13 (collagenase-3) was not changed. This mmp preferentially cleaves type II collagen, which is the major collagen type in articular cartilage, reflecting that this might not be related with the perifollicular tissues.

CD147 is an upstream inducer of mmps (Sameshima et al. 2000, Li et al. 2001, Si et al. 2003) that induces and modulates mmp expression, as the name suggests. Tang et al. (2004) and Gabison et al. (2005) suggested the presence of signal loops between CD147 and mmp in tumor and host cells. Tumor cells use cell surface CD147 to initiate contact with the surrounding fibroblasts, signaling them to synthesize mmps. The mmps secreted by fibroblasts then cleave the cell surface CD147 to generate soluble CD147. This soluble CD147, in turn, stimulates further mmp and CD147 expression via paracrine action. With this in mind, it is important to know the function of CD147 in tooth eruption. In this study, CD147, which has never been reported in tooth eruption, was expressed widely in follicular cells, osteoblasts and osteoclasts as well as in ameloblasts and odontoblasts. It was speculated that the CD147 secreted from odontogenic and follicular cells might induce mmp secretion through paracrine action to the follicular space and adjacent alveolar bone. In turn, perifollicular cells excrete mmps, which contribute to alveolar bone resorption and collagen digestion to facilitate tooth eruption. This hypothesis is supported by the present results in that the level of CD147 expression in the 2nd molar germs (at the root formation stage) was much higher than that in the 3rd molar germs (at the cap stage).

Bone resorption is a rate-limiting factor for the eruption of tooth germs in the alveolar bone, which can be accelerated or retarded by the local delivery of eruption factors. Although this study suggests a putative relation-

ship between CD147 and tooth eruption, further studies will be needed to determine the functional relationship using other direct ways, such as a knock-out model. Moreover, CD147 is a cyclophilin A receptor (Yurchenko et al. 2001, 2002) and is upregulated upon monocyte differentiation (Kasinrerk et al. 1999, Liang et al. 2002, Major et al. 2002). Therefore, further studies will be needed to understand the combined actions of cyclophilin A and its receptor, CD147, during tooth eruption. Furthermore, CD147 is involved not only in the induction of mmps but also in cell differentiation (reviewed by Huet et al. 2008). Hence, the involvement of CD147 in the differentiation of ameloblasts and odontoblasts cannot be excluded.

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어금니발생에서 이돋이 관련 물질 탐색: CD147과 바탕질분해효소

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간추림 : 발생기 치아 이돋이는 이돋이 관련 물질의 조절 아래 이루어지며 따라서 이돋이 관련 물질의 구명은 이 분야의 연구에서 주된 과제가 되어 왔다. 발생기 이돋이 관련 물질은 치아주머니세포로부터 유리되며 뼈파괴세포의 형성과 관여하는 것으로 알려져 있다. 그러나 이틀뼈를 포함한 주위 조직의 흡수가 이돋이경로 형성을 위하여 필수적이다. 따라서 치아주머니세포로부터 바탕질분해 관련 단백질이 분비되어 이돋이경로 형성에 관여하리라 가정된다. 또한 법랑질모세포와 상아질모세포가 이돋이 관련 물질을 분비하여 인접 조직에 영향을 미침으로써 이돋이를 도울 수 있다. 본 연구는 현재까지 치아에서 보고된 바 없는 바탕질단백질의 일종인 CD147과 바탕질분해효소의 치아 이돋이 과정에서 역할을 구명하고자 시행되었다. 출생 후 흰쥐 3, 6, 9일에서 발생기 위턱 둘째 및 셋째 어금니를 적출하여 면역조직화학염색과 RT-PCR을 이용하여 발현을 평가하였다. 발생중인 치아 상방에 출현한 뼈파괴세포는 이돋이 상태를 평가하기 위하여 조사되었다.

CD147 mRNA와 mmp-2 및 mmp-9 mRNA는 발생 9일에 뿌리형성기에 있는 둘째어금니에서 모자시기에 있는 셋째어금니보다 발현양이 훨씬 많았다. 또한 mmp-2 및 mmp-9 발현은 둘째어금니 발생 3 (후기 종시기), 6 (치아머리시기), 9일 (뿌리형성기)에 발생시간 의존적으로 증가되었다. 대조적으로 동일 시기에 mmp-13 mRNA 발현 변화는 관찰되지 않았다. 면역조직학적 관찰에서 CD147은 법랑질모세포와 상아질모세포 외에 치아주머니세포와 뼈파괴세포에서 강하게 발현되었다. 둘째어금니 교합면 위 이틀뼈에 출현한 뼈파괴세포 수는 뿌리형성기에서 유의하게 증가하였다.

이상의 결과로 보아 CD147은 mmp-2 및 mmp-9과 함께 치아 이돋이경로 형성에 관여할 것으로 생각된다.

찾아보기 낱말 : CD147, 이돋이, 바탕질분해효소

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