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Localization of Eruption-Related Molecules in Developing Rat Incisors

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A complex and intricate cascades of gene expression is necessary for tooth development, and tooth eruption is one of main events during tooth development. Tooth eruption is considered to be strictly controlled either by signaling or extracellular molecules that are secreted by the tooth germs themselves. There have been numerous reports on the eruption-related molecules; however, there is still a lack of information regarding the preparation of the eruption pathway and the key tissue factors involved. This study aimed to localize the eruption-related molecules such as cyclophilin A (Cyp-A), extracellular matrix metalloproteinase inducer (EMMPRIN), matrix metalloproteinase proteins (MMP-9), receptor activator of nuclear factor kappa-B ligand (RANKL), and osteoprotegerin (OPG) in the developing rat incisors.

Immunofluorescence findings revealed that Cyp-A, EMMPRIN, OPG, and RANKL were expressed strongly in the dental lamina epithelium of the rat incisor germs during eruptive movement. OPG was also expressed in the osteoclasts in the apical region. MMP-9, a final tissue resolution molecule, was more widely localized in the area surrounding the erupting incisor. This study suggested that the dental lamina epithelium may play a key role in preparing eruption pathways for the incisor in rats.

Keywords: Cyp-A, EMMPRIN, OPG, RANKL, MMP, tooth eruption

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Introduction

The developmental process of tooth germ begins with the dental lamina. The dental lamina epithelium invaginates into the lamina propria to develop an enamel organ. The developmental processes of tooth germs are subdivided crown formation (bud, cap and bell stages) and root formation stages with tooth eruption[1, 2]. The eruption of developing tooth germs is an occlusal movement to relocate them in appropriate position of the oral cavity. The processes of eruption comprise the resorption of the alveolar bone to make an eruption pathway and formation of the alveolar bone to fill in the space previously occupied by the tooth germs. Many crucial molecules such as cyclophilin A (Cyp-A), extracellular matrix metalloproteinase inducer (EMMPRIN), matrix metalloproteinase proteins (MMP), receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG) have been suggested to make eruption pathways, but their functional roles of where and how they involve in the eruption processes are largely unknown.

Cyp-A, EMMPRIN and MMP-9 play a major role in matrix resorption. Cyp-A is not only a potent chemoattractant for monocytes and macrophages which are necessary for the preparation of an eruptive pathway of tooth germs but also an EMMPRIN ligand[3, 4]. EMMPRIN is an internal membrane receptor that mediates the internalization of bound Cyp-A. It also stimulates the production of MMPs. MMPs stimulate tooth eruption pathway by degrading matrix components[1]. RANKL and OPG play an important role in osteoclasts function for bone resorption. RANKL promotes osteoclast formation, fusion, differentiation, activation, and survival, thus enhancing bone resorption [5]. Its biological effects are produced when it binds to RANK. OPG is a decoy receptor of RANKL and competes with RANKL for RANK binding. Thus, a RANKL/OPG ratio is important to determine final effects on bone resorption[6, 7].

Many studies have attempted to find molecules involved in the tooth eruption. However, there still has been a lack of information especially on how eruption pathway is prepared. Rodent incisors continue to erupt for their lives and can be a good model to investigate tooth eruption. This study was performed to determine the implication of several molecules in preparing an eruption pathway in rat mandibular incisors.

Materials and Methods

Animals and tissue preparation

Rat pups (Sprague-Dawley) were housed in laboratory animal care-approved facilities. All procedures were performed in accordance with the ethical standards formulated by the animal care and use committee in Chonnam National University. Immediately after rats at postnatal day 10 were sacrificed, portions of the mandible containing the incisor germs were isolated, immersion-fixed overnight in a 4% paraformaldehyde solution and decalcified with ethylene diamine tetra-acetic acid (pH 7.4). They were then dehydrated and embedded in paraffin. Five-μm-thick sagittal sections were cut serially for H-E staining.

Immunofluorescence staining

Immunofluorescence staining was performed using a TSATM kit (Invitrogen, Carlsbad, CA, USA). Briefly, after blocking the endogenous peroxidase with 1% H2O2, the deparaffinized sections were reacted over night with purified rabbit polyclonal anti-cyclophilin A (Calbiochem, San Diego, CA, USA) and purified goat monoclonal anti-EMMPRIN, MMP-9, OPG, RANKL (Santa Cruz biotechnology, Inc. Santa Cruz, CA, USA), respectively, followed by a reaction with the horseradish peroxidase-conjugated secondary antibody. The sections were incubated in a Tyramide working solution and counterstained using propidium iodide. The reactants were observed using a LSM confocal microscope (Carl Zeiss, Gottingen, Germany). The primary antibodies were substituted with normal serum for the negative control.

Results

Histological findings

At postnatal day 10, incisor germs were at the tooth eruption stage which was morphologically characterized by the completion of a crown and root outline and functionally by osteoclastic bone resorption for the preparation of an eruptive pathway. At all stages of tooth eruption, incisor germs were confined within the connective tissue of the dental follicle and bony crypts outside. The developing incisal ridge was covered by thick epithelial tissue of dental lamina, whereas apical region was surrounded in the dental sac or dental follicle (Fig. 1).

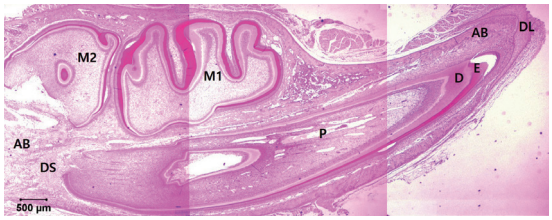

Fig. 1. The rat mandibular incisor germ is at the eruption stage of development processes. The incisal ridge was covered by thick epithelial tissue of dental lamina. AB: alveolar bone, D: dentin, DL: dental lamina, DS: dental sac, E: enamel, M1: 1st molar, M2: 2nd molar, P: pulp.

Immunofluorescence findings

Immunofluorescence staining of negative controls omitting the primary antibody did not show any reactivity (Fig. 2). Immunoreactivity against Cyp-A, EMMPRIN and MMP-9 were region-specific. Strong immunoreactivity against Cyp-A was found in the dental lamina epithelium of the eruption stage which is overlaying the incisal region of the developing incisor at postnatal day 10. Immunoreactivity was also seen in the alveolar bone matrix itself and perifollicular tissues and the reactivity was stronger in the incisal half than the apical half (Fig. 3). Immunoreactivity against EMMPRIN was stronger in the incisal half than the apical half of the germ. Strong immunoreactivity was found in the dental lamina and perifollicular cells (Fig. 4). Immunoreactivity against MMP 9 was weak at the dental lamina of the incisor. However, its reactivity was seen in wider area including alveolar bone and perifollicular tissues and developing enamel matrix (Fig. 5).

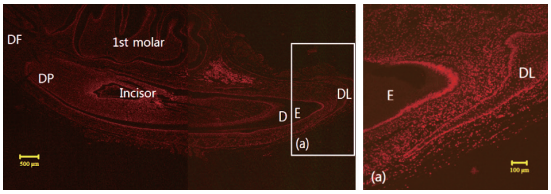

Fig 2. Negative control by immunofluorescence in the incisor germ at postnatal 10. D: dentin, DF: dental follicle, DL: dental lamina, DP: dental papilla, E: enamel

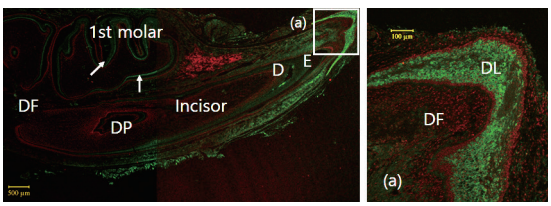

Fig. 3. Localization of Cyp-A by immunofluorescence in the incisor germ at postnatal 10. Strong immunoreactivity is found in dental lamina epithelium overlying the germ. Immunoreactivity is also seen in the follicular tissue and odontoblastic layers of molars (arrows). D: dentin, DF: dental follicle, DL: dental lamina, DP: dental papilla, E: enamel

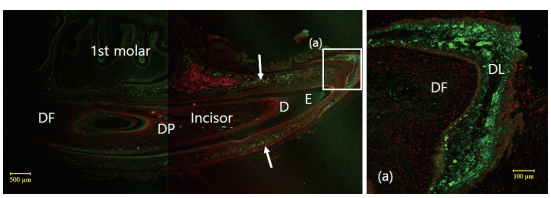

Fig. 4. Localization of EMMPRIN by immunofluorescence in the incisor germ at postnatal 10. Strong immunoreactivity is found in the dental lamina. Reactivity is also seen in perifollicular cells (arrows). D: dentin, DF: dental follicle, DL: dental lamina, DP: dental papilla, E: enamel

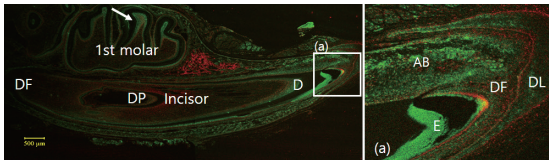

Fig. 5. Localization of MMP-9 by immunofluorescence in the incisor germ at postnatal 10. Immunoreactivity is weak the dental lamina of the incisor. However, reactivity is seen in wider area including enamel matrix, alveolar bone (AB) and odontoblasts (arrow). D: dentin, DF: dental follicle, DL: dental lamina, DP: dental papilla, E: enamel

Immunoreactivity against OPG was strong at the both apical and incisal region. Specifically, the reactivity was seen at osteoclasts at the apical region as well as the dental lamina epithelium of the incisal region (Fig. 6). Immunoreactivity against RANKL was strong in ameloblasts, in particular, in addition to perifollicular tissues (Fig. 7). Immunoreactivity of the investigated proteins in tissues was summarized in Table. 1.

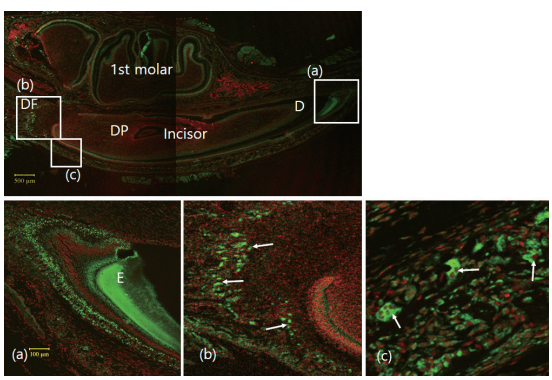

Fig. 6. Localization of OPG by immunofluorescence in the incisor germ at postnatal 10. Rectangular areas were magnified. Strong immunoreactivity is found in osteoclasts (arrows) as well as dental lamina epithelium overlying the germ. D: dentin, DF: dental follicle, DL: dental lamina, DP: dental papilla, E: enamel

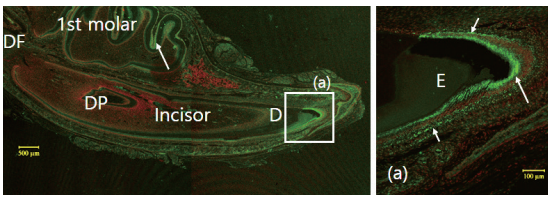

Fig. 7. Localization of RANKL by immunofluorescence in the incisor germ at postnatal 10. Strong immunoreactivity is found in ameloblasts (long arrows) and outer dental epithelial cells (short arrows) as well as dental lamina overlying the germ (DL). D: dentin, DF: dental follicle, DP: dental papilla, E: enamel

Table 1. Immunoreactivity of the investigated proteins

proteins	DL	AB	PFC	EM
Cyp-A	+++	++	++	-
EMMPRIN	+++	-	++	-
MMP	+	++	++	+++
OPG	+++	++	+	+++
RANKL	++	+	+	-

DL: dental lamina, AB: alveolar bone, PFC: perifollicular cells, EM: enamel matrix

Discussion

The eruption of tooth germs is an axial movement toward the oral cavity from their bony crypts in the developing alveolar bone [8, 9]. The movement begins soon after the formation of the root. In the present study, mandibular incisor germs of rats at postnatal day 10 were at active eruptive movement and this was evidenced morphologically by the presence of Hertwig epithelial root sheath and the appearance of many osteoclasts on the surface of alveolar bone [10].

Like other tooth development processes, tooth eruption is also undertaken by paracrine-mediated cells which communicate through signal molecules rather than direct cell-to-cell contacts. Signal molecules involved in tooth eruption are released from dental follicular cells and affect adjacent cells. Cyp-A was originally discovered as an intercellular ligand for cyclosporine A, an immunosuppressant, but other functions have been suggested. Its secreted form is a potent chemoattractant for macrophage colony-stimulating factor (MCSF)-dependent macrophages, monocytes, neutrophils, eosinophils and T cells [11]. This molecule is involved in the adhesion of monocytes /macrophages to the extracellular matrix [12] and the migration of murine bone marrow cells [13]. In addition, the secreted form modulates the osteoblastic activity [14] as well as the functions of endothelial cells via paracrine and autocrine modes [15]. In the present study, Cyp-A was expressed strongly in the dental lamina epithelia which covered the incisal region of the erupting incisors. Cyp-A is internalized by binding to the glycosylated cell surface receptor known as an EMMPRIN or basigin or neurothelin or CD147 [3]. The localization of EMMPRIN was paralleled to that of Cyp-A. This receptor is involved in the MMP expression. In the present study, EMMPRIN was localized mainly in the alveolar bone surrounding the incisal half of the incisors. Distribution of MMP-9 which is a final executive molecule for tissue digestion was wider in area, suggesting that perifollicular tissue adjacent developing incisors may actively involve in making eruption pathways.

Besides the destruction of the alveolar bone by osteoclasts, the resolution of organic matrix of the bone and surrounding connective tissue is a prerequisite for the formation of an eruption pathway. The resorption process of organic matrix is undertaken by MMPs [10]. The present findings also raised the possibility that Cyp-A, along with EMMPRIN, and MMP-9 may play a role in the maturation, resorption of developing enamel and dentin matrix. This study

raised a possibility that Cyp-A released from dental lamina may bind to EMMPRIN as its receptor and finally induced MMP-9 expression.

For tooth germs to erupt occlusally within the bony crypts which overlay them should be removed for the preparation of an eruption pathway. OPG, RANKL and its cognate receptor activator of nuclear factor kappa (RANK) function as a paracrine regulators of osteoclastogenesis and bone remodeling. OPG is a member of the tumor necrosis factor (TNF) receptor superfamily and a secretory soluble protein that lacks transmembrane and cytoplasmic domains. OPG is a decoy receptor and competes with RANKL for RANK binding. OPG protein not only inhibits terminal stages of differentiation, resorptive function of osteoclasts but also stimulate apoptosis of them [16]. Thus, bone resorption is modulated by a balance between RANK-RANKL binding and OPG production. In the present study, strong immunoreactivity against OPG in the incisor germs was seen in the alveolar bone surrounding the apical region of incisors. This regional specificity provided strong evidence that the OPG expression was positively involved in the eruption of the incisor germs.

RANKL is a 317 amino acid peptide. RANKL promotes osteoclast formation, fusion, differentiation, activation and survival, thus enhancing bone resorption as a key positive regulator of osteoclastgenesis. Its biological effects are produced when it binds to RANK, and neutralized by OPG which works as a soluble receptor antagonist and therefore prevents RANKL-RANK interaction [6, 17]. In the present study, strong immunoreactivity against RANKL was found in the dental lamina epithelium overlaying incisor germs at postnatal 10. This finding suggests that follicular cells at the tooth eruption stage may express RANKL proteins. Subsequently, released RANKL may play a role in the differentiation of osteoclasts which are involve in the alveolar bone resorption for the preparation of an eruption pathway.

In summary, Cyp-A, EMMPRIN, MMP-9 and RANKL are important for matrix resorption during eruption periods. Their immunoreactivity is strong or moderate in dental lamina epithelium. OPG is important to determine final effects on bone resorption. OPG immunoreactivity was found osteoclasts as well as dental lamina epithelium, implying its wide range of function during eruptive movement. Altogether, dental lamina epithelium may play an important role during the eruption stage of rat mandible incisors by secreting various signaling and extracellular molecules. Further studies are needed for their functional networks.

Acknowledgements

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한글초록

백서 절치 맹출 관련 인자 발현에 대한 면역형광염색 연구

한금동, 심해경, 김세은, 강지혜, 김민석, 이은주, 김선헌

전남대학교 치의학전문대학원 구강해부학교실

복잡하고 정교한 유전자 발현이 맹출 시기를 포함한 치아 발생 단계에 필요하다. 치아 맹출은 맹출 관련 유전자에 기반한 인자들에 의해 엄격히 조절되며, 맹출 인자들은 치배 자체에서 분비된다. 지금까지 맹출 관련 인자들에 대한 많은 연구들이 이루어 졌지만 여전히 어떻게 맹출이 일어나는 지에 대한 지식은 부족한 실정이다. 이번 연구는 백서 절치의 맹출 과정 동안 나타나는 신호 인자들을 확인하였다. 치아 맹출 중 Cyp-A, EMMPRIN, MMP-9은 기질 흡수에 중요한 역할을 하고, OPG 및 RANKL은 골 흡수 및 흡수 조절에 중요한 역할을 한다. 면역형광염색 결과 Cyp-A, EMMPRIN, RANKL이 맹출 시기에 치판상피에서 강하게 발현됨을 나타냈다. OPG는 치판상피 뿐 아니리 치근첨 주위에 출현한 파골세포에서도 강하게 발현되었으며, 조직분해 말기에 작용하는 MMP-9은 치배 주위 조직에 광범위하게 발현되었다. 이상의 결과는 백서 절치에서 치판상피는 맹출로 형성에 필수적인 요소임을 시사하였다.

주제어: Cyp-A, EMMPRIN, OPG, RANKL, MMP, 치아맹출