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Extracellular Matrix Metalloproteinase Inducer is Regulated Developmentally and Functionally in the Rat Submandibular Gland

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The salivary gland undergoes complex process of growth and differentiation of the branching morphogenesis of ductal system during the prenatal and early postnatal periods which are regulated by various elements in the extracellular matrix. Extracellular matrix metalloproteinase inducer (EMMPRIN) is a cell adhesion molecule. In the present study, localization and expression of EMMPRIN in development and effects of chorda-lingual denervation and cyclosporine A (CsA) treatment on the EMMPRIN expression were investigated. Immunohistochemistry, RT-PCR and Western blot were used to determine expression level. Immunohistochemistry revealed that EMMPRIN was localized specifically in the cytoplasm of ductal cells, not acini of the submandibular gland all the postnatal periods. At prenatal day 18, when the formation of ducts was not definite, no immunoreactivity was observed. Both Western blot and RT-PCR analyses revealed that EMMPRIN expression was maintained up to postnatal day 7, decreased after postnatal day 10. The EMMPRIN expression was upregulated by the surgical denervation of the chorda-lingual nerve in the gland as well as by the CsA treatment. The present study suggests that EMMPRIN is a crucial molecule for maintaining physiological functions of the salivary gland.

Key words: EMMPRIN, submandibular gland, chorda-lingual nerve, CsA

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Introduction

The development of the major salivary glands in mammals begins during the prenatal period and reaches completion at the early postnatal period [1]. The morphogenesis of the gland is a complex process, generating epithelial buds and their ramifications, and finely regulated by elements in the extracellular matrix and hormones or the autonomic nervous system [2]. The formation of a lumen within epithelial buds begins in the distal portion to generate ductal system. The proximal portion of epithelial buds ultimately develops into acini connected to the oral cavity by a rather complicated ductal system. The parenchyma of the mature submandibular gland consist of acini, intercalated ducts, granular convoluted tubules, striated ducts and the interlobular ducts that form common excretory ducts which lead to the oral cavity. At birth, the rodent submandibular gland continues to develop a ramifying ductal system which is composed of terminal tubular and proacinar cells. By postnatal day 2, the terminal tubules mostly consist of proacinar cells, which give rise to

acinar cells during the first week of development. At the end of the first week, the parenchymal cells further develop into predominant acinar cells and small portions of the terminal tubules, which become intercalated ducts in the future [3,4].

Cell adhesion molecules are intimately involved in a variety of cellular processes, including development, growth, apoptosis, and differentiation of cells. Interactions of these molecules with components of extracellular matrix and growth factors provide an intricate regulatory mechanism for a variety of cellular phenomena. Extracellular matrix metalloproteinase inducer (EMMPRIN, also called as CD147, basigin or neurothelin) has been regarded as a member of the immunoglobulin superfamily class of cell adhesion molecules. This protein is an integral membrane receptor, mediating internalization of bound cyclophilin which is a family of proteins from vertebrates that bind to cyclosporin A (CsA) [5]. CsA is an immunosuppressant drug widely used in organ transplantation to prevent rejection by lowering the activity of T cells and their immune responses.

EMMPRIN is an important cell surface molecule in the developmentally regulated biological process. The EMMPRIN-knockout mouse embryos cannot undergo implantation and have defects in sensory, immune and memory functions [6]. This molecule stimulates production of multiple matrix metalloproteinase as the name suggests. EMMPRIN is also an essential component for the cyclophilin-mediated signaling cascade on the presence of heparan sulfate, a subtype of glycosaminoglycan as primary binding sites [5]. Recombinant purified or soluble secreted EMMPRIN glycoprotein stimulates fibroblasts, producing high levels of matrix metalloproteinases (MMPs) such as MMP-1, MMP-2 and MMP-3 [7,8]. The importance of EMMPRIN is increasing from the perspective of its involvement in cancer progression in the salivary gland [9,10].

For the morphogenesis of the salivary gland, degradation and formation of matrix as well as cell proliferation in the gland is expected. In addition, atrophying by cutting nerves associated with salivary glands may alter molecular expression including EMMPRIN. We hypothesized that EMMPRIN may contribute to the morphological change in development and atrophy after denervation or CsA treatment. The present study was performed to describe the presence of EMMPRIN and its putative functions in the submandibular gland in rats.

Materials and Methods

Animals

Sprague-Dawley rats were brought up in Association for Accreditation and Assessment of Laboratory Animal Care-Approved Facilities and provided with regular food and tap water. Adult male and pup rats at prenatal day 18 and postnatal days 1, 4, 7, and 10 were used. All procedures were performed in accordance with the ethical standards of the animal care committee at Chonnam National University.

Denervation of the chorda-lingual nerve

Adult rats approximately weighing 200 g were anesthetized by intraperitoneal injection of ketamine (50 mg/kg). After a vertical incision was made along the main duct of the right submandibular gland, the chorda-lingual nerve was carefully exposed under a microscope at the level of the hypoglossal nerve which crosses the chorda-lingual, and severed under the mylohyoid muscle. The left side was sham-operated for the control. The salivary glands of both sides were removed at postoperative days 5 and 13.

Administration of CsA

Cyclosporine A (CsA, Chongkundang Pharm, Seoul, KOR) was dissolved in olive oil and subcutaneously injected everyday into postnatal day 1 rats at a concentration of 10 mg/kg for 10 days.

Tissue preparations and Immunohistochemistry

Adult submandibular glands were perfused with 4% paraformaldehyde solution and further immersion-fixed overnight. They were then dehydrated in ethyl alcohol and embedded in paraffin. Sections were cut 5 μm thick and stained with H-E for morphological findings.

Immunohistochemical reaction was performed using Vectastain Elite ABC Kit (Vector Laboratories, CA, USA). Purified polyclonal anti EMMPRIN (Santa Cruz biotechnology Inc., CA, USA) was used as a primary antibody. For negative control of the reaction, normal horse serum was substituted for the primary antibody. Blocking endogenous peroxidase and subsequent non-specific reactions were undertaken by incubating sections in 0.3% H_2O_2 and in a blocking serum respectively. Sections were reacted in the

primary antibody at 4℃ overnight, followed by incubation in biotinylated secondary antibody for 2 hrs. They were then reacted in avidine-biotin peroxidase complex and developed with AEC (3-Amino-9-ethylcarbazole) for microscopic observation.

RT-PCR

The isolated glands were immediately frozen in liquid nitrogen. Total RNA was extracted using a Trizol® Reagent (Invitrogen, CA, USA) according to manufacturer's instructions. The extracted RNA samples were quantified by UV spectrophotometer (Amersham-Pharmacia Biotech, Arlington Heights, IL, USA). EMMPRIN primers (GenBank accession no. BC 059145.1) were custom-designed. The sequences for the primer pair were 5' CGG AAT TCC GGA ACA CGC CAG TGA GG 3' for the forward and 5' GCG GAT CCA CAG GAG TGG AGG CAG ACG 3' for the reverse, generating an expected PCR product of 553 bp. The housekeeping gene GAPDH (GenBank accession no. NM_012783.1) was also amplified using primers of specific 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.

First strand cDNA was synthesized using Superscript II (Gibco BRL, MD, USA). RT controls were carried out using the same RT reaction mix except substituting the cDNA for DEPC-treated H_2O. PCR products were resolved on a 1.2% agarose gel and visualized using ethidium bromide. The size was confirmed using 1 kb DNA ladder (Gibco BRL, MD, USA).

Western blot

Total proteins were prepared from the isolated glands. Briefly, protein lysates were loaded into 12% SDS-polyacrylamide gel and electrotransferred to Hybond ECL membranes (Amersham Pharmacia Biotech, IL, USA). The membranes were then incubated with the polyclonal antibody against EMMPRIN (Calbiochem, CA, USA) and then incubated in horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, MA, USA). Immunodetection was performed using the ECL substrate (Amersham Pharmacia Biotech, IL, USA) according to the manufacturer's instructions. The protein markers were the Prestained SDS-PAGE Standard which is ranged from 6 to 194 kD (Bio-Rad, CA, USA).

Results

Histological Findings

At prenatal day 18, the parenchyma of the salivary glands mainly consisted of epithelial proacini, which were incompletely divided into lobules by connective tissue septa. However, ducts were very sparse in the parenchyma (Fig. 1A). At postnatal day 1, definite formation of ducts including intercalated ducts was observed (Fig. 1B). The ducts at postnatal day 10 increased in size and number through branching morphogenesis of ductal system (Fig. 1C), being similar to those of adult rats (Fig. 1D).

Immunohistochemical Findings

The submandibular glands were immunohistochemically stained for EMMPRIN. Immunoreactivity of EMMPRIN was not observed in developing glands at prenatal day 18 (Fig. 2A). Reactivity was seen in small ducts at postnatal day 1 (Fig. 2B). The staining intensities at postnatal day 10 (Fig. 2C) were stronger than those in the adult glands (Fig. 2D). The immunoreactivity was increased in the glands at 5 days after denervation of the chorda-lingual (Fig. 2E). Negative controls omitting primary antibodies were negative in reaction (Fig. 2F).

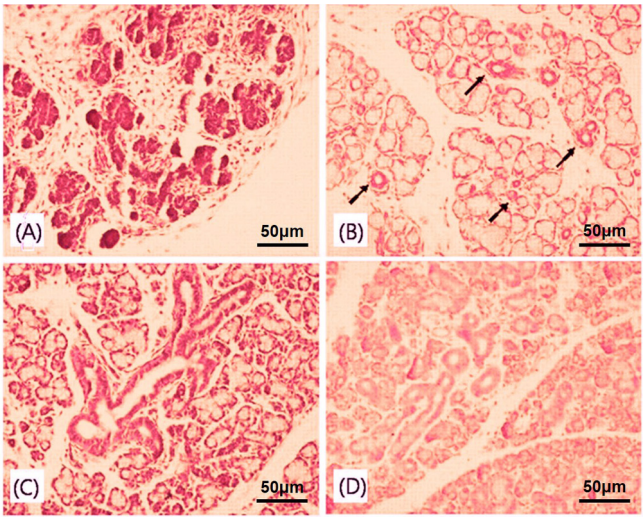

Fig. 1. Developmental features of the submandibular gland with H-E staining. (A) At prenatal day 18, developing islets of epithelial cells do not yet form definite ducts. (B) At postnatal day 1, small ducts (arrows) are developing through branching morphogenesis. (C) A hierarchial ramification of ducts is exhibited at postnatal day 10. (D) The submandibular gland in adult rats. Scale bars represent 50 ㎛.

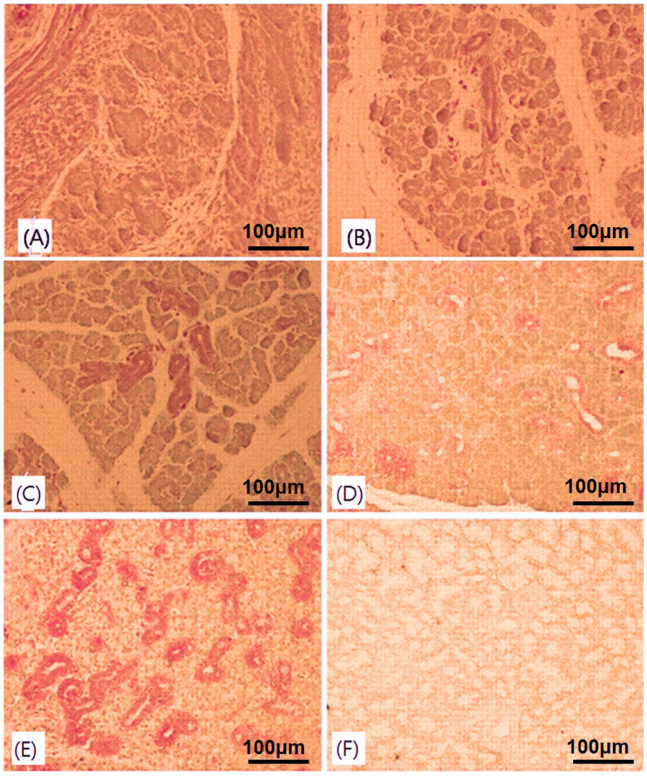

Fig. 2. Immunohistochemical staining against EMMPRIN through developmental stages. (A) Immunoreactivity is not observed in the developing glands at prenatal day 18. (B) The immunoreactivity is seen in small ducts at postnatal day 1. (C) The strong immunoreactive ducts at postnatal day 10. (D) The immunoreactivity in adult is weaker than that in the early postnatal days. (E) The immunoreactivity is increased in the glands at 5 days after denervation of the chorda-lingual. (F) A negative control omitting primary antibody is negative in reaction. Scale bars represent 100 ㎛.

EMMPRIN expression in the submandibular glands

To determine expression of EMMPRIN mRNA from the submandibular glands, RT-PCR was performed at 5 developmental time points including postnatal days 1, 4, 7 and 10 and adult. Expression of EMMPRIN mRNA was observed in a single band of 553 bp as expected. The expression was increased up to postnatal day 7 in a time dependent manner, and significantly decreased at postnatal day 10 and adult (Fig. 3A). This result was confirmed at a protein level by immunoblotting, with the same expression pattern (Fig. 3B).To determine the effect of denervation of the chorda-lingual nerve which contains secretomotor fiber in the submandibular glands, expression of EMMPRIN mRNA was investigated by RT-PCR at 5 and 13 days after the denervation. The effects of the denervation after postoperation day 13 were confirmed by comparing the size of the

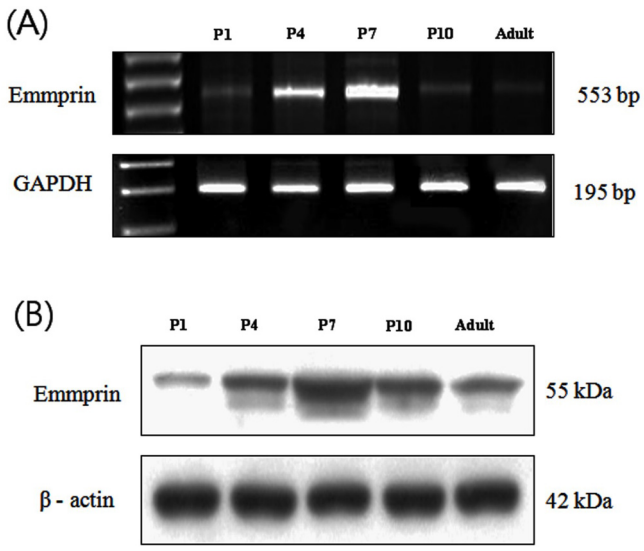

Fig. 3. RT-PCR and Western blot were performed to investigate the expression changes of EMMPRIN. (A) Expression of EMMPRIN mRNA was determined by RT-PCR. EMMPRIN was expressed at all the 5 developmental time points. (B) Expression of EMMPRIN protein was determined by Western blot at 5 different development time points.

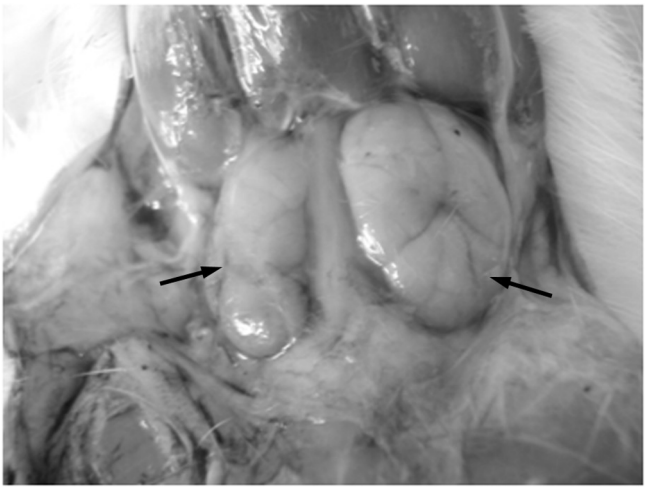

Fig. 4. The gland of the denervated right side (the left arrow) was approximately reduced half in size, comparing with the control at the contralateral left side (the right arrow) at day 13 after the chorda-lingual nerve cut.

normal gland with the cut submandibular glands. The glands of cutting right side were reduced approximately half in size, comparing with the normal at the left contralateral side (Fig. 4). Its expression level was increased by the denervation at both mRNA (Fig. 5A) and protein levels (Fig. 5B).

To determine the effect of CsA on the expression of EMMPRIN, its mRNA level was determined by RT-PCR at postnatal day 10 after CsA administration. Its mRNA level

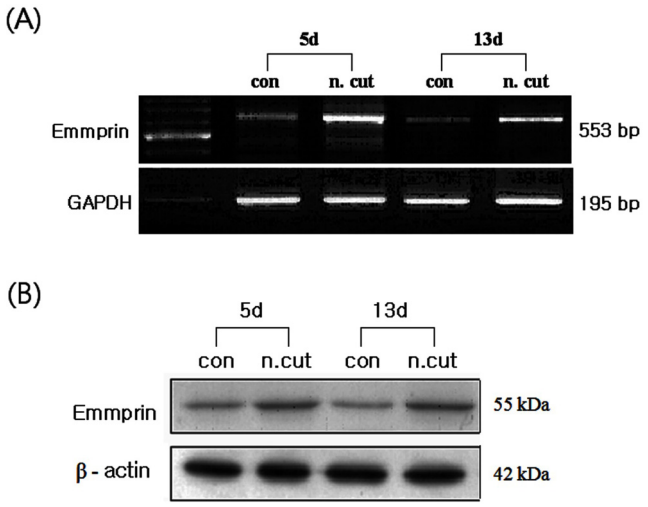

Fig. 5. RT-PCR and Western blot was performed to elucidate the effect of denervation of the chroda-lingual nerve on EMMPRIN expression. (A) Expression of EMMPRIN mRNA was increased by the denervation at post-operation days 5 and 13. (B) Expression of EMMPRIN protein was also increased by the denervation at both postoperative days 5 and 13.

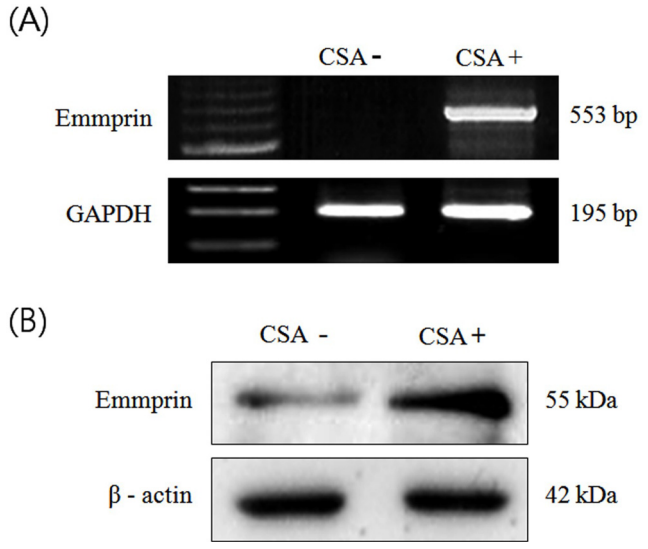

Fig. 6. RT-PCR and Western blot was performed to elucidate the effect of CsA on EMMPRIN expression. (A) Expression of the mRNA was increased by CsA treatment for 10 days. (B) Increased expression level of the protein was also confirmed by Western blotting.

increased by the administration (Fig. 6A). Also, this result was confirmed at the protein level by immunoblotting (Fig. 6B).

Discussion

The rodent salivary glands develop through a branching morphogenesis that is regulated by elements in the extracellular matrix, hormones and the autonomic nervous system. Previous studies have indicated that temporal and spatial coordination through stable cell-substratum interactions is needed for cytoarchitectural organizations in the salivary gland [11]. Development of the major glands in rodents begins at embryonic day 12 (E12) and continues postnatally. These glands are not readily distinguishable from each other until roughly E15 [12]. At birth, the salivary glands consist of ramifying ductal systems which end in transient tubular structures (terminal tubules), which give rise to acini and are the sites of future intercalated ducts [13]. We also observed that the ductal systems in submandibular glands were not formed before birth, and the morphogenesis was rapidly progressed until postnatal day 7 (Fig. 1A-D).

Little is known about molecular events which regulate the development of rodent salivary glands. The present study represented the first examination of the distribution and expression of EMMPRIN in the submandibular gland. EMMPRIN has been regarded as a member of the immunoglobulin superfamily class of cell adhesion molecules. This protein is an integral membrane receptor, mediating internalization of bound cyclophilin on the presence of heparan sulfate, a subtype of glycosaminoglycan as primary binding sites [5]. It is an important cell surface molecule in biological process and developmentally regulated. EMMPRIN on the surface of tumor cell stimulates the production of multiple matrix metalloproteinase such as MMP-1, MMP-2 and MMP-3 by adjacent stromal cells [7, 8].

The present study demonstrated EMMPRIN expression from submandibular gland at the levels of mRNA and proteins. Its localization was exclusively found in many ducts including the intercalated, striated and excretory ducts. The fact that EMMPRIN was expressed in these ducts suggests that EMMPRIN has a common functions of proliferation, migration and apoptosis in diverse ductal systems. The ducts in the salivary gland are not only passages for saliva but also sites for the modification of saliva composition. In the present study, the localization of EMMPRIN could not be found at prenatal day 18, when definite duct formation was not seen and septal compartment was not complete. From the immunohistochemical findings, expression of EMMPRIN was demonstrated at mRNA level at 5 different time points. The mRNA expression levels of EMMPRIN were increased up to postnatal day 7, afterwards

decreasing at postnatal day 10 and adult. These patterns of changes in expression were also confirmed at a protein level, suggesting that the molecule may be involved in other functions such as the developmental regulation of the gland. This result coincides with the report that demonstrated EMMPRIN is related in proliferation and migration in the salivary gland tumor [14].

To demonstrate that EMMPRIN is involved in other functions than saliva secretion, the present study designed a model which does not secrete saliva by surgical denervation of the chorda-lingual nerve, the secretomotor parasympathetic fibers. By the denervation, the affected side of the submandibular gland was remarkably reduced in size. The expression of EMMPRIN in the affected side was increased comparing with that of the contralateral side as control. Recently, Piao et al. repoerted that inreased EMMPRIN and MMP-9 is correlated salivary duct carcinoma [15]. These results suggested at least that the EMMPRIN may be involved in the matrix reconstruction by regulating matrix enzymes such as MMPs rather than the secretion of saliva .

CsA, a potent immunosuppressive agent is a hydrophobic cyclic peptide which is naturally produced by the fungus *Tolypocladium inflatum* [16,17]. This chemical forms a heterotrimeric complex with cyclophilin A which is internalized by the mediation of EMMPRIN [18,19]. CsA stimulates proliferation of thymic epithelial cells and their endocrine function in human as well as mice [20]. Also, CsA leads to functional and morphological changes of the exocrine glands such as salivary and lacrimal glands by reduction in the number of secretory granules and their aggregation [21]. In the present study, the EMMPRIN expression at both transcriptional and translational level was increased by the CsA treatment, suggesting that EMMPRIN may involve in CsA-induced functional changes of exocrine glands. This results are supported by the report that CsA and EMMPRIN is correlated with the recruitment of leukocyte in the inflammatory diseases [22].

Taken together, the present study demonstrated that EMMPRIN was localized in ducts of the salivary glands and its expression was regulated by experimental conditions. Its expression was decreased at adult, when the formation of ducts is apparently no longer active. These results also raised a possibility that the molecule be implicated in changes in ductal cells such as proliferation or differentiation [23,24] or protection of ductal cells from death [25] by mediating an internalization of bound cyclophilin as an integral membrane receptor. Moreover, the clinical involvement of EMMPRIN in the progression of adenoid cystic carcinoma has been found to be important [26], concise function of EMMPRIN in the morphogenesis of ducts and matrix changes in the salivary gland should be further explored.

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Conflict of interest

The authors declare that they have no competing interest.

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