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APin expression during amelogenesis

조선대학교 대학원

치의공학과

이 창 섭

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법랑질 형성과정 중에 Apin의 발현

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ABSTRACT

APin expression during amelogenesis

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This investigated the expression and localization of APin cDNA, which was previously identified and cloned from a rat odontoblast cDNA library, during ameloblast differentiation in rat incisors using *in situ* hybridization and immunohistochemistry. The subcellular localization of APin varied during ameloblast differentiation but was stage-specific. APin mRNA was not expressed in the preameloblasts, was weakly expressed in the secretory ameloblasts and strongly expressed in the maturation stage ameloblasts as well as in the junctional epithelium attached to the enamel of molars. In the maturation stage ameloblasts, APin protein was strongly expressed in the supranuclear area (Golgi complex) of the smooth-ended ameloblasts as well as in both the supranuclear area and the ruffle end of the ruffle-ended ameloblasts. During the ameloblast-lineage cell culture, APin was expressed at a low level in the early stages, but at a high level in the late stage of culture,

which was equivalent to the maturation stage. APin protein was efficiently secreted from transfected cells in culture. Furthermore, its over-expression and inactivation caused an increase and decrease in matrix metalloproteinase-20 (MMP-20) and tuftelin expression, respectively. These findings indicate a functional role for APin in the mineralization and maturation of the enamel that is mediated by the expression of MMP-20 and tuftelin.

Key words: APin, enamel, ameloblast differentiation, enamel maturation

Ⅰ. *INTRODUCTION*

Enamel formation is a complex and well-coordinated biological processinvolving two major steps, secretion and maturation. During the secretory stage, tall columnar ameloblasts actively synthesize and secrete the enamel matrix proteins through Tomes'processes. These proteins include amelogenin (1, 2), ameloblastin (3, 4), enamelin (2), and tuftelin (5, 6). Non-amelogenin enamel proteins, such as ameloblastin, enamelin, and tuftelin, are believed to be nucleators of hydroxyapatite crystal formation during enamel mineralization (7, 8, 9). In addition, MMP-20 (enamelysin), which plays an important role in the degradation of amelogenin, is synthesized and secreted (10, 11). The amelogenin degradation induced by MMP-20 is believed to be essential for the axial growth of enamel crystals (12, 13). During the maturation stage, low columnar ameloblasts become less active in the synthesis and secretion of enamel matrix proteins. However, they synthesize and secrete Kallikrein-4 (KLK4), which degrades enamel proteins to promote enamel crystal thickening (14, 15). During this stage, ameloblasts develop either a ruffle- or smooth-end that plays an important role in the mineralization and maturation of enamel by removing water and the enamel matrix degradation products as well as transporting calcium (16, 17). Although there have been advances in our understanding of enamel formation, further studies will be needed to better understand the precise mechanism of enamel mineralization and maturation.

The cloning of OD-314 cDNA from a rat odontoblast cDNA library and its expression in odontoblasts was previously reported (18). The cDNA was subsequently found to code for the APin protein, which was recently isolated as a secreted component of the amyloid deposits of a human calcifying

epithelial odontogenic tumor (CEOT) (19). *APin* was also reported to be one of the genes strongly expressed in gastric cancer by the serial analysis of the gene expression (SAGE) data (20). Recently, APin was identified from the secretome profile of the rat enamel organ based on a signal trap methodology, as well as an acidic protein rich in glutamine and proline that is strongly expressed in maturation stage ameloblasts (21).

In situ hybridization (ISH) and immunohistochemistry (IH) were performed in order to more precisely define the temporo-spatial expression of APin during ameloblast differentiation. In an initial step of investigating the role of APin in enamel formation, this study examined the effects of APin over-expression and inactivation on the expression of the enamel matrix protein mRNA in ameloblast-lineage cells (ALC) *in vitro* (22) using a reverse transcriptase polymerase chain reaction (RT-PCR), real-time PCR, and Western blot analysis. These studies show that APin is strongly expressed in maturation stage ameloblasts and that its expression is linked to the expression of MMP-20 and tuftelin, which are involved in enamel mineralization and maturation.

Ⅱ. *MATERIALS AND METHODS*

All experimental procedures involving the use of animals were reviewed and approved by the Animal and Human Subjects Research Committee at Chosun University of Korea.

Sequencing and sequence comparison of the OD-314 clone

The RNA from the odontoblasts/pulp cells of rat mandibular incisors was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) and converted to cDNA by reverse transcription (18). The full length OD-314 cDNA was obtained by determining the 5′ and 3′ unidentified sequences using a RACE kit (Roche, Mannheim, Germany), employing 2 μg of the total RNA from the odontoblasts/pulp cells as a template. The gene specific primers used for RACE were GSP1 (5′-CCACATAGGACATAGGACTAGCCTGCTG) and GSP2 (5′-GCAATTTCAGAGCGCCTTCAACTCCTGG). The 5′- and 3′-RACE products were ligated into pCR2.1-TOPO (Invitrogen) and sequenced with M13F (-47) and M13R (-48) primers. The resulting sequences were compared with the sequences in the DNA databases using BLAST (NCBI).

Histology, in situ hybridization (ISH) and immunohistochemistry (IH)

Sprague-Dawley rats (1, 16 and 41 d old) were perfused with 4% paraformaldehyde in PBS. The mandibles were removed, decalcified and processed for embedding in paraffin. Six-μm thick mesio-distal serial sections of the mandibular incisors (1 and 16 d old rats) and molars (41 d old rats) were cut, de-paraffinized and stained with hematoxylin and eosin (H&E) for histology, or were subjected to either ISH or IH.

For ISH, the digoxygenin (DIG)-labeled APin sense and antisense cRNA probes were prepared from the OD-314 cDNA using a T7/Sp6 DIG RNA labeling kit (Roche), as described previously (23).

For IH, the APin specific antibodies were obtained by affinity purification of the APin antisera that had been produced by immunizing rabbits with a synthetic peptide (Peptron, Seoul, Korea), STSPKPDTGNF, corresponding to the sequence, of 241 through to 251 of the 278-residue rat APin. The titre of the APin antibodies was determined using an enzyme-linked immunosorbent assay (ELISA) before being used for Western blotting and immunohistochemistry. To localize APin, a biotin-labeled goat anti-rabbit IgG (1:200) was used to bind to the rabbit anti-rat APin antibody (0.2 μg/ml) and the biotin was detected using the ABC kit (Vector, Burlingame, CA, USA). Rabbit preimmune serum was used as a control and the sections were counterstained with hematoxylin.

Cell culture and RT-PCR

Immortalized ALCs were kindly provided by Dr. T. Sugiyama (Akita University School of Medicine, Akita, Japan) and cultured as previously described (22). They were cultured for 3 d on type I collagen-coated culture dishes containing MEM supplemented with 10% FBS, antibiotics (Invitrogen) and 10 ng/ml of the recombinant human EGF (Sigma-Aldrich, St. Louis, MO, USA) (growth media). In order to investigate the expression of the enamel matrix proteins and enzymes during ameloblast differentiation, the confluent ALCs (day 0) were cultured for up to 4 wk in high-glucose DMEM supplemented with the same components (differentiation media).

For RT-PCR analysis, the total RNA from the ALCs was isolated using

TRIzol (Invitrogen) and used for cDNA synthesis and PCR amplification. The Primer sequences used were: *APin*: 5′-ccagcaggctagtcctatgtcctatgtgg/5′-cgcgtcgacatgagatcagtg, *amelogenin*: 5′-ccagagcatgataaggcagc/5′-gaactggcatcattggttgc, *ameloblastin*: 5′-aaaaggagaaggtccagaag/5′-tgcggaaggatagtaagtgt, *enamelin*: 5′-gacctatgccatgatgcctg/5′-cgctgataacggctgagtgt, *tuftelin*: 5′-acaaaccctttataggagcc/5′-aattaaaatttgggcctacc, *MMP-20*: 5′-agctgtgagcaactgatgactgga/5′-acagctagagccaagaacacacct, *KLK4*: 5′-aggagatgaggcagggaaga/5′-gttcccctgctctggcttac, and *GAPDH*: 5′-accacagtccatgccatcac/5′-tccaccaccctgttgctgt (forward/reverse). *GAPDH* was used as the reference gene for normalization. The following PCR conditions were used: denaturation at 94℃ for 5min, followed by 35 cycles each of denaturation at 94℃ for 30 s, primer annealing at 60℃ for 30 s, and product extension at 72℃ for 30 s, with a final extension at 72℃ for 7 min. The PCR products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light.

Construction of APin-expressing and APin siRNA-expressing plasmid

The coding portion of APin was PCR amplified from the rat full length OD-314 cDNA (APin cDNA) as a template. The PCR product was cloned into the EcoRI site in the sense orientation of pcDNA3 (Invitrogen), a eukaryotic expression vector. The potent cytomegalovirus promoter (pCMV) was inserted into the HindIII site of pcDNA3. Expression of APin was driven by the pCMV in the ALCs.

Based on the chosen 19-nucleotide APin siRNA sequence (5′-AAGTGCCTCAAGATCAAAC) using the "siRNA Target Finder and design

Tool″ (Ambion, Austin, TX, USA), APin siRNA-expressing plasmid was prepared using the p*Silencer* 1.0-U6 siRNA expression vector (Ambion) according to the manufacturer's instructions.

Tansfection and real-time PCR

The ALCs were plated (2 × 105^5 cells/ 60-mm dish) and cultured for 24 h. Then the cells showed approximately 50-60% cell confluence. The Cells were transiently transfected with 2 μg of the pCMV driven APin-expressing plasmid, U6-APin siRNA-expressing plasmid, and the control empty vector (Mock). Transfections were performed using the Lipofectamine Plus (Invitrogen) according to the manufacturer's protocol. The experiments were performed three times in duplicate.

At 6 h after transfection, the cells were incubated for 42 h in a growth media supplemented with 5% FBS. At 48 h after transfection, the expression of the mRNAs for *APin, amelogenin, ameloblastin, enamelin, tuftelin, MMP20* and *KLK4* were assessed by RT-PCR using the same primer and PCR conditions as described above.

For real-time PCR, specific primers of *APin, tuftelin, MMP20* and *GAPDH* were designed based on rat mRNA sequences. The Primer sequences used were: *APin*: 5′- aacactagagagctttgctgggct /5′- agatggtgtctgctgctgtgagaa, *tuftelin*: 5′- agcagaacagagtaatgtggccct/5′- tgacagtcagcgttcttgatccga, *MMP-20*: 5′- tgtctaagctcaaggtgccctgtt/5′- taagttgtccatgtgggtgctgga, and *GAPDH*: 5′- tccagaacatcatccctgcctcta/5′- acaaagtggtcgttgagggcaatg (forward/reverse). Setting these primers as a guide of Cybergreen Premix Sol (Bioneer, Seoul, Korea), the expression quantity of *Apin, MMP-20, tuftelin* and *GAPDH* were confirmed by Exicycler (Bioneer). Significance of the observed △△Ct values

was confirmed statistically by two-tailed Student's t-test. The relative amount of each mRNA was determined at 50% levels of PCR product, and normalized with use of the relative amount of GAPDH mRNA.

Fluorescence microscopy and Western blotting

The pCMV driven APin-expressing plasmid was transfected into C2C12 cells with the Lipofectamine Plus (Invitrogen) as described above, in six-well culture plates for Western blots or Lab-Tek chambered coverglasses (Nunc, Rochester, NY, USA) for fluorescence microscopy. Cells were treated with 0.1% DMSO as vehicle alone or 10 μg/ml brefeldin A (Sigma-Aldrich).

For fluorescence microscopy, the cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with Triton X-100 in PBS, and quenched with NH_4Cl (50 mM) in PBS. Cells were incubated for 1h with the anti-APin antibody, washed with PBS, and then incubated for 1 h with a fluorescent-labeled secondary antibody (Vector). The cells were counterstained with PI (propidium iodide) to visualize the nucleus. After rinsing, the cells were examined with fluorescence microscope (Olympus, Tokyo, Japan).

Western blotting analysis was performed to analyze APin expression. The proteins were extracted from the cell lysates after the cells had been lysed in a NP-40 lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% NP-40, 2 mM Na_3VO_4, 2 mM $Na_4P_2O_7$, 50 mM NaF, 2 mM EDTA, pH 7.4, 10 μg/ml leupeptin, and aprotinin). The conditioned media were also collected and centrifuged at 14,000 RPM for 5 min at 4℃ to remove dead cell debris. The supernatant were precipitated for 1 h on ice with 10% trichliroacetic acid. The precipitated proteins were lysed with lysis buffer as described above. Samples were loaded on a denaturing 10-20% Tris-HCl polyacrylamide gel and

transferred to nitrocellulose membranes. APin was detected using the anti-rabbit APin antibody (1:1500), goat anti-rabbit-IgG (1:10,000), and ECL detection system (Amersham Pharmacia Biotech., Piscataway, NJ, USA).

Ⅲ. *RESULTS*

Sequence analysis of full-length cDNA of rat OD-314 (APin) clone

The full-length rat OD-314 cDNA had an identical nucleotide sequence to that of the rat *APin* (Genebank accession no. DQ198380). The cloned 1191 bp cDNA contained the entire 834 bp *APin* open reading frame, the 47 bp of 5′ untranslated region and the 310 bp of 3′ untranslated region (data not shown). The open reading frame encoded a 278-residue protein.

Expression of APin mRNA and protein during ameloblast differentiation in vivo

The continuously erupting mandibular incisors of the rat exhibited the sequential differentiation of ameloblasts: presecretory ameloblasts in the proximal region, mature and post maturation stage ameloblasts in the distal region, and secretory ameloblasts between them (Fig. 1A, C). ISH was performed with an APin-specific cRNA probe to determine if the APin transcript appears during ameloblast differentiation. APin mRNA was expressed weakly in the secretory ameloblasts (Fig. 1B, 2G) but strongly in the maturation ameloblasts (Fig. 1B, 2H, I) and the junctional epithelium (Fig. 2J), which originated from post-maturation stage ameloblasts. However, no APin mRNA was expressed in the preameloblasts and papillary cells (Fig. 1A).

IH was carried out using affinity purified rabbit APin antibodies to determine the subcellular location of APin protein during ameloblast differentiation. Interestingly, the subcellular localization of APin was varied but was stage-specific during ameloblast differentiation. APin was not expressed in preameloblasts, weakly expressed in secretory ameloblasts (Fig. 1C, 2D), and

strongly expressed in the maturation (Fig. 2E, F), post-maturation stage ameloblasts (Fig. 1C) and junctional epithelium bodering the enamel of the first mandibular molar (Fig. 2L). In the secretory ameloblasts, APin was expressed weakly in the cytoplasm and Tomes′ processes, but strongly on the enamel matrix deposited adjacent to the Tomes′ processes (Fig. 2D). The localization of APin in the maturation stage ameloblasts was more significant, with APin being detected strongly in the supranuclear region (Golgi complexes) of the smooth-ended ameloblasts (Fig. 2E) as well as in both the supranuclear region and the ruffle end of the ruffle-ended ameloblasts (Fig. 1C, 2F).

APin and enamel matrix gene expression during ALC differentiation in vitro

In order to determine the expression of APin, enamel matrix proteins and enzymes during ALC differentiation *in vitro*, the cells were cultured in the differentiation medium for up to 4 wk and their level of mRNA expression was assessed by RT-PCR. The selective and time-dependent induction of the enamel matrix proteins and enzymes was observed during ALC differentiation (Fig. 3A). APin and KLK4 mRNA expression gradually increased, whereas amelogenin, enamelin, andtuftelin mRNA transcription gradually decreased with cell differentiation (Fig. 3A). The expression of MMP20 mRNA increased slightly from the beginning of the culture to 7 d, and decreased thereafter. However, ameloblastin mRNA expression remained unchanged throughout the culture period. A similar APin protein expression pattern was observedby western blot using a polyclonal anti-rabbit APin antibody (Fig. 3B).

Effect of APin over-expression and inactivation on enamel matrix gene expression in vitro

In an attempt to understand the function of APin in enamel formation, the effect of APin over-expression and inactivation on the expression of the mRNAs of the enamel matrix proteins and enzymes *in vitro* was examined by RT-PCR and real-time PCR. APin expression increased and decreased markedly after its over-expression and inactivation, respectively (Fig. 4A). Over-expression of APin in the ALCs slightly up-regulated the expression of tuftelin and MMP-20 mRNA, whereas their expression was markedly down-regulated by its inactivation (Fig. 4A). However, the expression of the other enamel matrix proteins and enzymes remained unchanged when the APin was either up- or down-regulated (Fig. 4A).

Real-time PCR was also performed to confirm the relative level of APin, tuftelin and MMP-20expression after over-expression and inactivation of APin. APin mRNA was detected after 2 cycles of PCR in over-expression group and 17 cycles in inactivation group. In control and Mock group, the expression started after 7-8 cycles of PCR (Fig. 4B). The differences of Ct values were detected (data not shown). Consequently, over-expression of APin in ALCs up-regulated the expression of tuftelin and MMP-20, whereas siRNA inactivation of APin markedly decreased their expressions.

Subcellular localization of APin protein and their secretion in culture media

Immunofluorescence was performed after transfection with the APin-expressing plasmid into C2C12 cells. APin protein was clearly localized to crescent like structures mostly in the cytoplasm (Fig 5A). The relative transfection efficiency

was approximated visually and was confirmed by a second investigator. APin was barely expressed in normal C2C12 cells. A conservative estimate showed that at least 60% of C2C12 cells were APin-positive when transfected with the APin-expressing plasmid (data not shown).

Western blotting was performed to investigate the secretion of APin in culture media. The protein detected by Western blotting in the media migrated near their predicted molecular weight, around 30 kDa. When brefeldin, an inhibitor of protein secretion, was added, the APin protein was not detected in the conditioned media of transfected cells (Fig 5B). Expression of APin showed a strong signal, around 30.6 kDa, in the total cell lysate. Interestingly, when bredfeldin was added, there were two signals of APin protein, around 30.6 and 30 kDa, in the cell lysate (Fig 5B).

Ⅳ. *DISCUSSION*

In a previous study, we documented the potentially odontoblast-specific/enriched rat cDNA clones obtained by suppression subtractive hybridization (18). The expression of one of the clones, OD-314, in an odontoblastic cell culture was verified by Northern blotting and was further characterized (18). The OD-314 clone showed abundant transcripts in odontoblast/pulp cells by Northern blotting. A predominant transcript at ~ 1.2 kb and a much fainter transcript at ~2.2 kb in size were detected. These transcripts showed a pattern similar to that described for the EO-009 in the rat incisor enamel organ (21). In this study, the nucleotide and deduced amino acid sequencing of the full-length OD-314 cDNA revealed that it was identical to the sequencing of the rat EO-009cDNA encoding a 278-residue protein. A portion of deduced human EO-009 protein (residues 127-279), conserved in rat and mouse, was identical to a protein named APin (21). Consequently, we now refer the OD-314, gene product as *APin*. Although the analysis of the deduced amino acid sequence of the full length *APin* (EO-009) revealed the presence of a signaling peptide (21), the APin protein from CEOT-associated amyloiddoes not have a signal peptide at its N terminus (19). Therefore, it was postulated that a 153-amino acid APin protein was derived from a longer secreted precursor protein (21).

In preliminary studies we observed expression of APin in ameloblasts and that the expression was much higher in maturation stage ameloblasts compared to odontoblasts (data not shown). This study showed the presence of APin in the enamel matrix deposited adjacent to the Tomes′ processes during the secretory stage. These findings collectively indicate that APin is a secretory protein that is synthesized and secreted by secretory ameloblasts. Interestingly,

among the oral mucosa examined, only the junctional epithelium expressed APin. This unique APin expression in the junctional epithelium and maturation/postmaturation stage ameloblasts supports the notion that the origin of the junctional epithelium is the reduced enamel epithelium (24, 25). Therefore, APin, along with amelotin (26) can be regarded as a marker for the junctional epithelium to differentiate it from the sulcular, gingival and other epithelia in the oral mucosa. It would be interesting to determine if the junctional epithelium, unlike other epithelia, has any other unique function(s) in maintaining the enamel surface.

When the ALCs were cultured for up to 4 wk in the differentiation media, they differentiated and sequentially expressed the enamel matrix proteins and enzymes as shown during ameloblast differentiation *in vivo*. The ability of the ALCs to express several ameloblast specific genes *in vitro* indicates that ALCs are relatively undifferentiated and undergo differentiation under the culture conditions. Therefore, ALCs are an invaluable cell line that can be used to examine the regulatory mechanisms of ameloblast differentiation and enamel matrix formation (22). We found that APin was expressed first by ALCs at 4 d with the highest level at 28 d, which is equivalent to the maturation stage. Over-expression of APin in the ALCs slightly up-regulated the expression of tuftelin and MMP-20 mRNA, whereas their expression was markedly down-regulated by its inactivation. It strongly suggests that APin regulate the expression of MMP-20 and tuftelin. However, in both cases, the expression of the other enamel matrix proteins remained unchanged. In this study, APin-expressing plasmid was transiently transfected into ALCs showing 50-60% cell confluence, and the expression of APin and the other enamel matrix proteins were evaluated at 48 h after transfection, which is equivalent

to the confluent stage (day 0) during ALCs differentiation. It implies that the cells could not reach the differentiation to express the enamel matrix gene yet.

In this study, we investigated if APin was in fact secreted from the cultured cells. Mouse myogenic C2C12 cells were chosen due to the absence of endogenous APin mRNA expression, as determined by RT-PCR (data not shown). The cells transfected with the APin-expressing plasmid showed high level of peptide in the cytolsol, as observed by fluorescence microscope. After transfection the proteins detected by Western blotting in the cell lysate and the media migrated at their predicted molecular weights, i.e. 30.6 and 30 kDa, respectively. The protein detected in the cell lysate had a slightly slower migration behavior as compared to that observed in the culture media. This suggests the secreted APin was post-translationally modified. However, it will be of great importance to exactly determine if these two APin protein products show different locations either in the extracellular enamel matrix as a secretory protein or in the intracellular locations such as the supranuclear area and the ruffle end.

Enamel mineralization and maturation are achieved through two major steps. During the secretory stage, ameloblasts secret MMP-20, which is involved in an extracellular enzymatic cleavage of amelogenin into small peptides prior to their removal by endocytosis (27, 28). One apparent function of proteolysis during the secretory stage is to produce the space for the axial crystallite growth (29). This is facilitated further by the removal of water and enamel matrix degradation products during the maturation stage, allowing the mineralization and maturation of the enamel continue until enamel formation is completed. The maturation ameloblasts also synthesize and secrete KLK4, which degrades the remaining enamel matrix degradation products (15),

contributing to crystallite thickening and hardening of the enamel (14). Furthermore, maturation stage ameloblasts cycle between ruffle- and smooth-ended phenotypes, which both have well-developed Golgi complexes containing numerous Golgi cisternae, lysosomes and multivascular bodies (16). The ruffle end, a zone of infolded cell membrane, is characteristically formed during phases of calcium transport and when the resorption of degraded enamel matrix occurs (30, 31). The localization of APin in the ruffle end, but not on the smooth end, of maturation ameloblasts indicates that APin may function in these processes. Although the staining in the ruffle end may represent degraded APin being internalized, as observed for degraded amelogenin products, amelogenin is not found in the supranuclear area (32). Thus, the intense staining for APin in the supranuclear area together with the strong signal for APin mRNA in the maturation ameloblasts indicates that APin is highly expressed in these cells and as a consequence the staining of APin in the ruffle end is more likely to relate to a function in calcium transport, which is associated with the presence of ruffle-ended ameloblasts (33, 34, 35). This notion is supported by the ability of recombinant APin to bind to EF-hand calcium binding proteins parvalbumin (unpublished data) using protoarray. The EF-hand calcium-binding protein parvalbumin could buffer calcium specifically in the cells producing mineralized enamel and dentine during the later stages of tooth development (36).

In summary, although further studies will be needed to understand the precise function of APin in amelogenesis, these results suggest that APin is involved in the mineralization and maturation of the enamel, possibly by regulate the expression of MMP-20 and tuftelin and/or aiding in the transporting calcium during the maturation stage.

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Ⅵ. *FIGURE LEGENDS*

Fig. 1. Low magnification micrographs showing APin expression during ameloblast differentiation in the mandibular incisor (1 d old rat) by *in situ* hybridization (1A and B) and immunohistochemistry (1C). (A) Showing the strong APin mRNA expression in the maturation and postmaturation stage ameloblasts (arrow). (B) Showing the weak APin mRNA expression in secretory ameloblasts (S) and the strong expression in smooth-ended (SE) ameloblasts shown in the rectangular area shown in Fig. 1A. (C) Showing the APin protein localization in secretory ameloblasts (S) and the strong expression in the smooth-ended (SE) as well as the ruffle-ended (RE) ameloblasts shown in an area marked with two lines in Fig. 1A. Scale bar, 20 μm.

Fig. 2. High magnification micrographs of incisor tissue sections from 16 d old rats after H&E staining (A, B, C), immunohistochemical localization of the APin protein (D, E, F), and in situ hybridization of APin mRNA (G, H, I) in the secretory (A, D, G), smooth-ended (B, E, H) and ruffle-ended (C, F, I) ameloblasts. (A) Showing the Tome's processes (arrow). The insert in "C" shows the ruffle-end (arrow) at high magnification. Note the weak APin localization in the cytoplasm and Tomes' processes (arrow) of secretory ameloblasts (D), contrasting the strong staining in the enamel matrix deposited adjacent to the Tomes' processes. The strong APin staining is evident in the supranuclear region (thick arrow) of smooth-ended ameloblasts (E), as well as in the supranuclear region (thick arrow) and ruffle-end (arrow) of the ruffle-ended ameloblasts (F). Note the weak APin mRNA expression in the secretory ameloblasts (G) but the strong expression in smooth- (H) and

ruffle-ended (I) ameloblasts.

ISH of APin mRNA (J) and IH of APin protein (L) in the junctional epithelium attached to the enamel of the mandibular molars (41 d old rat). Note the strong APin mRNA expression in the junctional epithelium of the interdental area between the first (M1) and second (M2) mandibular molars (J), but not in the control tissue (K). APin protein is specifically localized in the junctional epithelium bordering the enamel of the first mandibular molar (thick arrow), but not in the sulcular epithelium (arrow, L). AB, ameloblasts; PL, papillary layer; En, enamel. Scale bar, 20 μm.

Fig. 3. RT-PCR analysis of mRNA expression for enamel matrix proteins and enzymes during ALC differentiation *in vitro*. (A) Note the strong APin expression at 7 d and thereafter (equivalent to the maturation stage). (B) Western blot analysis of the expression pattern of APin during ALC differentiation *in vitro*. The expression of the APin protein appeared at 4d and continuously increased thereafter.

Fig. 4. RT-PCR (A) and real-time PCR (B, C) analysis of the effect of APin over-expression and inactivation on mRNA expression for enamel matrix proteins and enzymes. The ALC Cells were transiently transfected with the pCMV driven APin-expressing plasmid, U6-APin siRNA-expressing plasmid, and the control vector. (A) APin over-expression and inactivation changes the expression of tuftelin and MMP-20. (B) The amplification curves for the APin reactions in control, Mock, over-expression, and inactivation group. The relative amounts of tuftelin (C) and MMP-20 (D) mRNA, after normalization with the amounts of the GAPDH mRNA. The results were represented as means ± SD

of three independent transfections. The asterisks (*) indicate the values significantly different from the controls according to the Student's t test ($P < 0.01$). Control, normal ALC; Over, APin over-expression; inact, APin inactivation; Mock, ALC expressing empty vector.

Fig. 5. Subcellular localization of APin protein by immunofluorescence (A) and their secretion into culture media (B). The C2C12 cells were transiently transfected with the APin-expressing plasmid. (A) Cells were labeled with an anti-APin antibody and examined by fluorescence after counterstaining with PI. (B) The cell lysates (lanes 1-2) and conditioned media (lanes 3-4) were analyzed for the presence of APin protein by Western blotting. Expression of APin showed a strong signal in "Over" and two signals in "Over + Brefeldin A" of the cell lysates. Apin protein was detected as a strong signal in "Over" of the conditioned media, while the addition of brefeldin A caused no specific signal in the conditioned media. PI. Propidium iodide; Over, APin over-expression; Over + Brefeldin A, APin over-expression and Brefeldin A treatment.

Ⅵ. *FIGURES*

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

ABSTRACT in KOREAN

법랑질 형성과정중에 *APin*의 발현

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이 연구에서는 흰쥐 절치의 법랑모세포 분과과정 중에 전에 흰쥐 상아모세포에서부터 분리된 APin의 발현을 *in situ* hybridization과 면역조직화학적방법 이용하여 연구하여 다음과 같은 결과를 얻었다.

APin의 세포내 발현(subcellular localization)은 법랑모세포 분화과정 중에 다양하게 나타났다. APin mRNA는 법랑모세포전구세포(preameloblasts)에서는 발현이 되지 않았고, 분비기 법랑모세포(secretory ameloblasts)에서는 약하게 발현되었으며, 구치의 법랑질에 부착된 부착상피(junctional epithelium) 뿐 아니라 성숙기의 법랑모세포(maturation stage ameloblasts)에서 강하게 발현되었다. 성숙기 법랑모세포에서 APin 단백질은 ruffle-ended 법랑모세포의 supranuclear area와 ruffle end 뿐 아니라 smooth-ended 법랑모세포의 supranuclear area (Golgi complex)에서 강하게 발현되었다. 법랑모세포주(ACL) 배양 동안에 APin의 발현은 배양 초기에는 약하였으나 성숙기에 해당되는 배양의 말기에는 강하게 발현되었다. APin 단백질은 배양한 transfected cell로부터 효과적으로 분비되었으며, 더더욱 그의 과발현은 matrix metalloproteinase-20과 tuftelin의 발현을 증가시켰고, 발현억제는 이

들의 발현을 감소시켰다.

위의 결과들은 MMP-20과 tuftelin의 발현에 의해 조절되는 법랑질의 석회화와 성숙에 있어서 APin의 기능적 역할을 암시한다.

Key words: APin, 법랑질, 법랑모세포 분화, 법랑질 성숙

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본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(O) 반대( )

2006년 12월 일

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