IMMUNOCHEMICAL AND IMMUNOCYTOCHEMICAL STUDY OF A 15 kDa NON-AMELOGENIN AND RELATED PROTEINS IN THE PORCINE IMMATURE ENAMEL: PROPOSAL OF A NEW GROUP OF ENAMEL PROTEINS 'SHEATH PROTEINS'

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ABSTRACT

Our previous immunohistochemical study showed that in the porcine immature enamel, 13-17 kDa non-amelogenins were preferentially localized in the prism sheath. In this study, we revealed that the N-terminal amino acid sequence of a 15 kDa protein, one of the main components of the 13-17 kDa non-amelogenins, was VPAFPRQPGTHGVASL-, which shows no homology to those of known enamel proteins. In immunochemical analysis of porcine immature enamel, antibodies against a synthetic peptide containing this sequence reacted with many proteins of different molecular weights, especially with 62 kDa, 40 kDa and 10-19 kDa proteins. Of these proteins, the 62 kDa protein of the highest molecular weight was found only in newly formed enamel. In immunohistochemical preparations of the porcine tooth germs, the immunoreactivity of immature enamel just beneath the nonsecretory face of the Tomes' processes was intense. This immature enamel seemed to grow into the prism sheaths which showed intense immunoreactivity throughout the entire thickness of immature enamel at the matrix formation stage. The Golgi apparatus and secretory granules of the secretory ameloblasts showed immunoreactivity. These results suggest that the 62 kDa protein is a parent protein of the low molecular weight non-amelogenins. From its unique amino acid sequence and localization, we propose the low molecular weight non-amelogenins and their parent protein comprise a new family of enamel proteins designated as 'sheath proteins'.

Fully mature enamel is a unique and highly mineralized tissue composed of enamel prisms, interprismatic substance and prism sheaths. The prism sheaths are known to be low-mineralized structures located between individual enamel prisms or interprismatic substance, both of which are highly mineralized structures (3). It has been shown that the shape of enamel prisms is defined by the behavior of secretory ameloblasts and their Tomes' processes, a portion of secretory ameloblasts proximal to the basal terminal bars (3). However, the mechanisms through which prism sheaths develop have not yet been elucidated.

Glimcher *et al.* showed that proteins contained in the prism sheaths of immature bovine enamel were different from those contained in the enamel prisms and interprismatic substance (8). Recently, we found that proteins reactive with the antiserum against the 13-17 kDa non-amelogenins were localized predominantly in the prism sheath, and that this antiserum reacted with neither amelogenins nor enamelines (6, 18). Therefore, we named these proteins 'sheath protein' for convenience (6). However, electrophoresis showed that the 13-17 kDa non-amelogenin fraction used for the production of the antiserum contained at least four dif-

ferent protein species (18). It has been impossible to identify the parent protein(s) of the 13–17 kDa non-amelogenins, although our results suggested that these molecules were degradation products. Moreover, we could not determine whether they were derived from a single parent species or a number of closely related proteins.

In this study, we determined N-terminal amino acid sequence of a 15 kDa protein, one of the main components of the 13–17 kDa non-amelogenins, and raised an antibody against the N-terminal region of this protein using a corresponding synthetic peptide. Immature enamel of the porcine tooth germs was investigated immunochemically and immunocytochemically to elucidate the parent protein(s) of the 15 kDa protein and the localization of these proteins.

MATERIALS AND METHODS

Purification of Enamel Protein and N-Terminal Sequence Analysis

The surface layer of immature enamel at the matrix formation stage was collected from developing permanent incisors of 6-month-old piglets as described previously (4). The enamel samples collected were homogenized in 0.05 M carbonate buffer (pH 10.8) containing several kinds of proteinase and phosphatase inhibitors. After centrifugation, the supernatants were concentrated by ultrafiltration and fractionated by gel filtration using Sephadex G-100 (Pharmacia Biotech, Tokyo) in the same carbonate buffer. The first elution peak, which contained low molecular weight non-amelogenin proteins (4), was separated into five fractions by a molecular sieve method using a tandem column of TSKgel G4000PW (Toso, Tokyo) equilibrated and eluted with 4 M guanidine HCl, 30 mM Tris buffer (pH 7.0), as described previously (6). The proteins eluted in the fifth peak (fraction V) was further fractionated by reversed-phase high performance liquid chromatography (HPLC, LC-3, Shimadzu, Tokyo) using a column (RESOURCE RPC 3 ml, Pharmacia Biotech) equilibrated with 0.05% trifluoroacetic acid. Chromatography was conducted using ascending acetonitrile concentrations from 0 to 80% in 0.05% trifluoroacetic acid. Each of the peak fractions was collected and lyophilized. The purification of the peak fraction corresponding to the 15 kDa protein was performed by rechromatography using the same system, until a single band was obtained on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 9).

The partial sequence of the N-terminal of the purified protein was determined using a protein sequencer (Applied Biosystem Model 477A, CA) in conjunction with reversed-phase HPLC (Applied Biosystem Model 130A).

Production of Antibodies

A heptadecapeptide including residues 1 to 16 from the N-terminal (see Results) of the 15 kDa protein was assembled using a Biolynx 4170 automatic peptide synthesizer (LKB, Uppsala, Sweden) and purified by reversed-phase HPLC using an ascending acetonitrile concentration as described previously (17). The purified peptide coupled to bovine serum albumin (BSA) was emulsified with Freund's complete adjuvant (first injection) or incomplete adjuvant (booster injections) and used to immunize rabbits. After three or four injections, blood was harvested and the serum was isolated by centrifugation. Specific antibodies were purified further on an affinity column of purified synthetic peptide coupled to amino-cellulofine (Chiso, Tokyo, Japan).

Immunochemical Analysis

Two types of enamel sample were taken from the matrix formation stage of developing permanent tooth germs of 6-month-old piglets. Outer layer enamel samples were collected from the outermost portion (about 30 μ m thick) of the immature enamel with a razor blade by the previously reported procedure (5, 17). Inner layer enamel samples were obtained from the immature enamel near the dentinoenamel junction. These samples were solubilized in excess 0.5 M acetic acid. After centrifugation, supernatants were concentrated by ultrafiltration and electrophoresed on a 12% polyacrylamide gel containing 0.1% SDS under nonreducing conditions (9). Sample proteins were transferred on GVHP membranes (Millipore, Yonezawa, Japan) according to the method of Towbin *et al.* (15). The membranes were immunostained by the avidin-biotin complex method as described previously (17, 18). The anti-peptide antibodies were used at a concentration of 4 μ g/ml.

Immunocytochemistry

Immunocytochemical analysis was performed as described previously (17). Briefly, deciduous tooth germs of piglets (both sexes, 2.5–3.5 kg body

Fig. 1 SDS-PAGE of fraction V eluted from a tandem column of TSKgel G4000PW equilibrated and eluted with 4 M guanidine HCl-30 mM Tris buffer, pH 7.0 (a), and of purified 15 kDa protein (b).

weight) were perfusion-fixed with either 4% paraformaldehyde plus 1% glutaraldehyde or 4% paraformaldehyde containing 0.4% picric acid and 0.2% glutaraldehyde in a sodium phosphate buffer, pH 7.4. After decalcification with EDTA, they were dehydrated in graded N,N-dimethylformamide at progressively lower temperatures and embedded in glycol methacrylate (GMA) at -20°C (16). Thick sections ($1\text{ }\mu\text{m}$) were cut and stained with toluidine blue to select desired areas. For light microscopy, ultrathin sections mounted on gelatin-coated glass slides were processed for immunogold-silver staining. The concentration of specific antibody was $4\text{ }\mu\text{g/ml}$ and the silver intensification time was about 30 min. The sections were mounted with Entellan (Merck) without counter staining. For immunoelectron microscopic analysis, ultrathin sections were mounted on nickel grids. The concentration of specific antibody was $1\text{--}2\text{ }\mu\text{g/ml}$ and the silver intensification time was chosen to achieve optimal silver deposition (about 6 min). The sections were counterstained with uranyl acetate followed by lead citrate and examined with a Hitachi H-300 electron microscope.

Immunohistochemical specificity was checked

Fig. 2 SDS-PAGE (a) and immunoblot analysis (b) of enamel samples obtained from porcine immature enamel. O, outer layer enamel sample; I, inner layer enamel sample

by the absorption test. Diluted antibody was preincubated overnight at 4°C with purified synthetic peptide ($20\text{ }\mu\text{g/ml}$) before the antibody was applied to the tissue sections.

RESULTS

Biochemical and Immunoblotting Analysis

Fig. 1 shows electrophoretic patterns of the fraction V fractionated by the TSKgel G4000PW column (Fig. 1a) and the purified 15 kDa protein (Fig. 1b) on a 15% polyacrylamide gel containing 0.1% SDS under nonreducing conditions. After iterative application to the RESOURCE RPC 3 ml column, the 15 kDa protein fraction appeared as a single band (Fig. 1b).

The N-terminal amino acid sequence of the 15 kDa protein was Val-Pro-Ala-Phe-Pro-Arg-Gln-Pro-Gly-Thr-His-Gly-Val-Ala-Ser-Leu-, which did not correspond to the reported sequences of amelogenins (1, 7, 10–14, 19), enamelines (Fukae *et al.*, unpublished data) or tuftelin (2).

Fig. 2 shows the electrophoretic (Fig. 2a) and

Fig. 3 Low magnification light micrographs of toluidine blue-stained (a) and immunostained (b) sections from a deciduous incisor, showing overall localization of immunoreactivity at the matrix formation (F), transition (T), and maturation (M) stages. Immunoreactivity of the surface layer of immature enamel (E) is markedly decreased at the transition stage (arrow), whereas that of the inner layer is intense until the early maturation stage (asterisk). D, dentin. $\times 80$

Western blot patterns (Fig. 2b) of the proteins extracted from the enamel samples. On Western blots, several protein bands ranging from 62 kDa to less than 10 kDa were stained by the anti-peptide antibody. Among these, the 15–19 kDa proteins were the main bands not only in the outer layer but also in the inner layer enamel sample. In the outer layer enamel sample, 62 kDa and 40 kDa protein bands and the diffuse protein band with a molecular weight of 30 to 35 kDa showed positive immunoreactivity. On the other hand, in the inner layer enamel sample, these 62 kDa and 40 kDa protein bands were not observed. Instead, a diffuse band with a molecular weight of 25–35 kDa and bands less than 15 kDa were more intense in the inner layer enamel sample. Comparison of these staining patterns showed that immunoreactive components

decreased in molecular weight from the outer enamel to the inner enamel. The 89 kDa protein band corresponding to enamelin and 21–25 kDa protein bands corresponding to amelogenins were not reactive with the anti-peptide antibodies.

Light Microscopic Immunocytochemistry

In light microscopic immunohistochemical preparations, immunoreactivity was weaker when the fixative used contained high concentrations of glutaraldehyde. However, similar staining patterns were obtained regardless of the fixative used.

Fig. 3 shows the overall distribution of positive immunoreaction in the deciduous incisor. Comparison of the thick section stained with toluidine blue for the histological observation (Fig. 3a) and

Fig. 4 Light micrograph of thicker enamel at the matrix formation stage from a molar tooth germ. Immunoreactivity shows a honeycomb pattern over the entire thickness of the enamel matrix (E). Arrows indicate the Golgi apparatus in the secretory ameloblasts (Am). Dentin (D) showed no specific staining. $\times 520$

the immunostained section (Fig. 3b), both of which were obtained from the same resin block, clearly demonstrated the localization of immunoreactivity. Enamel matrix at the stage of matrix formation, transition, and early maturation was stained by the anti-peptide antibodies (Fig. 3b). Just below the nucleus, differentiating and secretory ameloblasts contained weakly immunoreactive

structures, possibly corresponding to the Golgi apparatus (Fig. 4). No immunoreactivity was detected in the ameloblasts at the stages of transition and maturation.

At the stage of matrix formation, the immunoreactivity over the prism sheath of immature enamel was intense, producing a distinct honeycomb pattern (Fig. 4). This pattern was present through-

Fig. 5 Immunoreactivity in the extracellular matrix at the differentiation stage from a molar tooth germ. Immunoreactivity is detected over stippled material (arrows) and amorphous dense material (asterisks) containing electron-lucent clefts. Am, differentiating ameloblast. $\times 33,000$

out the full thickness of immature enamel. However, from the surface of the enamel matrix to a depth of about $20\ \mu\text{m}$, immunoreactivity over the enamel prism was moderate and thus the honeycomb pattern was less clear (Fig. 4). The immunoreactivity of enamel prism was decreased gradually toward the dentinoenamel junction. Deeper than $20\ \mu\text{m}$ in the enamel matrix, immunoreactivity over the enamel prism was weak and, therefore, the honeycomb pattern was very clear (Fig. 4).

At the transitional stage, the immunoreactivity of the immature enamel beneath the ameloblast disappeared, while the underlying layer showed intense immunoreactivity (Fig. 3b). At the maturation stage, immunoreactivity of the immature enamel decreased gradually from the surface layer, and disappeared before the enamel matrix was solubilized by EDTA decalcification (Fig. 3b). Dentin itself and dentinal tubules showed no immunoreactivity (Fig. 4).

Electron Microscopic Immunocytochemistry

At ultrastructural resolution, a similar localization of immunoreactivity was observed regardless of the fixative used.

At the differentiation stage, immunoreactivity

was detected initially in the differentiating ameloblasts and in the extracellular matrix at the stage when the basal lamina disappeared and biomineralization of predentin has occurred. In the differentiating ameloblasts, a few silver grains were observed over the Golgi apparatus, which was usually located on the distal or lateral aspect of the nucleus. Rough-surfaced endoplasmic reticulum (rER) was usually not labeled. A very few grains were present on the nuclei, mitochondria and cytoplasmic matrix. In the extracellular matrix at the differentiation stage, silver grains reflecting immunoreactivity were initially observed over the irregular mass of stippled material and small patches of amorphous dense material (Fig. 5) which appeared to give rise to a continuous layer of the initial enamel at the beginning of the matrix formation stage. Few grains were present on the dentin matrix.

In the secretory ameloblasts at the matrix formation stage, the Golgi apparatus and some secretory granules were labeled moderately (Figs. 6 and 7). Large lysosome-like granules were labeled occasionally (Fig. 6). The stippled material found in the intercellular spaces and along the plasma membrane of the Tomes' process also showed positive immunoreactivity. At the early stages of matrix formation, when Tomes' processes were not yet developed, immunoreactivity of the enamel matrix showed a gradient decreasing toward the dentinoenamel junction. After development of the Tomes' processes, the labeling of immature enamel was the same as that seen at the light microscopic level. There was a thin layer showing intense immunoreactivity along the nonsecretory face of the Tomes' processes (Fig. 7). This layer was rarely observed at the bottom of Tomes' processes, becoming gradually broader toward the top and finally connected with the prism sheath at the tip of the Tomes' processes. From the surface of the enamel matrix to a depth of about $20\ \mu\text{m}$, immunoreactivity over the enamel prism was moderate but showed a decreasing gradient toward the dentinoenamel junction. Deeper than $20\ \mu\text{m}$, the immunoreactivity over the enamel prism was markedly decreased, whereas that over the prism sheath was remained intense (Fig. 8). No specific relationship was found between immunoreactivity and apatite crystals in the enamel matrix (Figs. 5 and 7).

At the transition and maturation stages, no specific labeling was found over the ameloblasts including membrane-bound accumulations of fine granular material at the distal ends of the cells. The pattern of immunostaining over the enamel matrix

Fig. 6 Golgi region of a secretory ameloblast from a molar tooth germ. The Golgi lamellae (GL), Golgi vesicles and secretory granules (SG) show positive immunoreactivity. Large lysosomal spherical bodies (Ly) with various electron densities are also stained. Nucleus (N), rough endoplasmic reticulum (ER) and cytoplasmic matrix are immunonegative. $\times 32,000$

at the transition and maturation stages was the same as that seen by light microscopy.

Dentinal tubules in the mantle dentin contained amorphous dense materials which showed no immunoreactivity. Furthermore, no immunoreactivity was detected at any stage in the organelles of either the odontoblasts or the cells of the stratum intermedium.

Specificity Control

Immunostaining of the immature enamel was disappeared almost completely after preabsorption of the antibodies with purified peptide.

DISCUSSION

The present study showed that, in the outer layer

enamel samples, antibodies generated against a synthetic peptide fragment from the N-terminal region of a 15 kDa non-amelogenin protein reacted mainly with 62 kDa, 40 kDa and 15–19 kDa proteins. Among these proteins, the 62 kDa protein had the highest molecular weight protein and was not detected in inner layer enamel. In addition, cell organelles involved in production and secretion of proteinaceous substances in secretory ameloblasts showed immunoreactivity. Therefore, we conclude that the 15 kDa protein is likely to be a degradation product of the 62 kDa protein, which is secreted directly from secretory ameloblasts.

We have shown previously that the physical properties and amino acid compositions of the 13–17 kDa non-amelogenins are very similar to one another (4). In this study, antibodies specific to the N-terminal region of the 15 kDa protein reacted

Fig. 7 Tomes' process of a secretory ameloblasts from a molar tooth germ. Intense immunoreactivity (arrowheads) adjacent to the nonsecretory face (NF) of the Tomes' process connects with that over the prism sheath (asterisk). Some secretory granules (SG) show immunoreactivity. SF, secretory face of the Tomes' process. $\times 20,000$

with all these proteins, indicating that the 13–17 kDa non-amelogenins share the same or closely related epitope(s). At present, we can not exclude the possibility that the 13–17 kDa non-amelogenins are not related but contain similar antigen determinant(s) by chance. However, it seems more likely that 13–17 kDa non-amelogenins are produced from a common parent protein and, therefore, that their amino acid sequence is partially identical.

The immunoreactivity of the anti-peptide antibody at the matrix formation stage was detected over the enamel prism, interprismatic substance and prism sheath in the surface layer of the immature enamel. On the other hand, in the inner layer, immunoreactivity over the prism sheath was intense, although that over the enamel prism and interprismatic substance was very weak. On West-

ern blots, the most noteworthy differences between outer and inner layer enamel samples was that the 62 kDa and the 40 kDa proteins found in the outer layer enamel could not be detected in the inner layer enamel. These results suggest that the 62 kDa and/or 40 kDa proteins are contained in the enamel prism and interprismatic substance located near the secretory ameloblasts, while lower molecular weight immunoreactive proteins (degradation products) including the 15 kDa protein are localized in the prism sheath throughout the entire thickness of the immature enamel.

Electron microscopic immunocytochemical analysis showed that 140 kDa and/or 89 kDa enamelin are concentrated along the putative secretory face of the Tomes' processes through an unknown mechanism (17). In this study, immunoreactivity along the nonsecretory face of the Tomes' pro-

Fig. 8 Electron micrograph of inner layer enamel of the matrix formation stage from a molar tooth germ. Prism sheaths are stained intensely (arrows). $\times 13,000$

cesses increased gradually toward the dentinoenamel junction and was more intense than that along the putative secretory face. Therefore, there may be another mechanism through which the 13–17 kDa non-amelogenins, which are not originally secreted proteins but are degradation products, are concentrated along the nonsecretory face of the Tomes' processes. Further studies are needed to clarify the mechanism of this increase.

We have previously speculated that the 13–17 kDa non-amelogenins belong to a new category of enamel protein because of their unique amino acid composition (4), their characteristic localization in the prism sheath, and their putative ability to form prism sheaths (18). This speculation is reinforced by the present results that the N-terminal amino acid sequence of a 15 kDa non-amelogenin had no resemblance to known enamel proteins such as amelogenins and amelins, and that the 13–17 kDa non-amelogenins share common or closely related epitope(s). Recently, we demonstrated that the results of immunochemical and immunohistochemical analysis of bovine immature enamel using antiserum against porcine 13–17 kDa non-amelogenins were very similar to those of the pig (6), suggesting the possibility that the 13–17 kDa non-amelogenins are not specific to porcine immature enamel but are common to mammalian imma-

ture enamel. Indeed, the 13–17 kDa non-amelogenin antiserum reacted with the prism sheath of the immature enamel of cat and monkey (Uchida *et al.*, unpublished data). Although at present we have no other data such as cDNA sequence, the present findings together with those of previous studies suggest that the 13–17 kDa non-amelogenins, their putative parent protein and other immunologically related proteins may comprise a new family of enamel proteins, designated as 'sheath proteins'.

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