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Disturbed Enamel Biomineralization in col1-caPPR Mouse Incisor

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Abstract

During the mineralization process of enamel, gene expression controls the activities of ameloblasts, the secretion and assembly of an extracellular protein matrix, affecting the final structure and functions. In this study, the enamel in the maxillary and mandibular incisors of wild-type and transgenic (col1-caPPR) mice, in which a constitutively active PTH/PTHrP receptor (PPR) was targeted to osteoblastic cells, was observed by scanning electron microscopy (SEM), Fourier transform infrared microscopy (FTIRM), and nanoindentation. The SEM studies showed that several different patterns of aberrations in crystal arrangement, disturbed prism organization without decussation, as well as abnormal enamel distribution were encountered in transgenic enamel. FTIRM analysis revealed poorer crystallinity/maturity after mutation. Nanoindentation measurement disclosed that transgenic enamel had 24.6% lower hardness and 12.3% lower elastic modulus. We attributed the inferior properties to the loosely packing crystals and abnormal prism organization. Furthermore, the col1-caPPR mouse model was substantiated to be useful to study how genes modulate the biomineralization process.

Keywords

Constitutively active PTH/PTHrP receptor; Transgenic mouse; Enamel distribution; Biomineralization; Nanomechanical property

Enamel is a hierarchically ordered bioceramic tissue, and its development involves typical biomineralization, in which a synthesized extracellular organic matrix induces and governs the subsequent mineral deposition and organization under genetic control. It is characterized by four stages: secretion, transition, maturation, and mature tissue [1]. Enamel formation begins at the early crown stage of tooth development and involves differentiation of the cells of the internal dental epithelium at the tips of the cusp to ameloblasts under special gene control. As the ameloblasts migrate outward, they leave behind a ribbon of secreted proteins in their wake [2]. These proteins undergo self-assembly to form an enamel extracellular organic matrix that is capable of controlling the initiation, rate of growth, and organization of the hydroxyapatite crystals or other octacalcium phosphate precursors [3]. Subsequently, these inorganic crystallites are originally seeded and elongated in the initial enamel matrix [4]. As older matrix is degraded and resorbed, the crystals grow in width and thickness and almost entirely replace the organic phase during maturation [5, 6]. Thus, the biomineralization process is under the strict control of genes and cells from the initial developmentally regulated initiation of the matrix proteins through completion of the mature enamel [7]. Gene regulation serves to govern which proteins are expressed and where they are secreted to form an enamel extracellular matrix, while the ameloblasts control the conformation and direction of movement of secretory Tome's processes, which in turn dictates the orientations of the protein matrix [3, 7]. It is of importance to understand their functional roles in enamel mineralization, to provide insight into the factors controlling the formation and growth of crystals and how mutation in the mineralization process would lead to alterations or diseases.

Parathyroid hormone (PTH) is a major regulator of calcium homeostasis, while PTH-related protein (PTHrP) is an epithelial factor mediating epithelial–mesenchymal interactions, resulting in the development of many structures [8]. They bind to and activate the same PTH/PTHrP receptor (PPR) with almost indistinguishable high affinity and play an important role as an autocrine/paracrine factor in tooth development. The incisors studied here were from *col1-caPPR* mice described previously, in which a constitutively active PTH/PTHrP receptor was targeted to osteoblastic cells [9, 10]. Previous studies have detailed the marked histological changes [10]; however, little attention had been paid to their biomineralization process, especially their enamel microarchitectures, and concomitant chemical and mechanical properties, which are still obscure. It is necessary to relate the chemical and mineral properties to the information about the precise nature and relative timing of the histological changes. The present work, behind our general approach to the transgenic incisor enamel, was an attempt to bridge the gap between histological and chemical information by analyzing crystal organization and enamel distribution at a level appropriate to the distinctive anatomy and histology of the tissues. Moreover, we analyzed the movement of ameloblasts during incisor amelogenesis, especially for recognition of factors controlling mechanical and mineral properties as well as enamel structures.

The purpose of this study was to test the hypothesis that the *col1-caPPR* mutation, with subsequent alterations in protein matrix, would perturb nano-scale and meso-scale properties of enamel crystals and their assembling patterns and, thus, alter the prism distribution as well as mechanical properties. Differences in the enamel distribution and mineral properties between the wild-type (WT) and transgenic (TG) mouse incisors were studied by scanning

electron microscopy (SEM), Fourier transform infrared microscopy (FTIRM), and nanoindentation.

Materials and Methods

Sample Preparation

Eight WT and TG sex-matched littermates at 12 weeks of age were kindly provided by the Department of Medicine, University of Rochester School of Medicine [9, 10]. A total of 32 incisors were extracted and carefully cleaned of soft tissue before analysis.

Eight mandibular incisors in each strain were embedded in polymethylmethacrylate (Beijing Fine Chemical, Beijing, P. R. China), allowed to cure overnight in air at room temperature, then ground longitudinally or transversely [11, 12]. The embedded samples were cut following the schematic diagram, as shown in Fig. 1. The transverse sectioned/ground plane was located in the mature incisor enamel. All sectioned planes in TG enamel ground longitudinally or transversely were consistent with those in WT, to avoid the effect of a gradient in enamel maturation in an apico-incisal direction. Then they were metallographically ground with silicon carbide abrasive paper of decreasing grit size under deionized water (320, 600, 1,200, and 2,500 grit) and polished on microcloths with successively finer grades of alumina powder. During polishing, the specimens were ultrasonically cleaned in deionized water for 2 minutes after each grade of polishing to remove surface debris and finally stored in the refrigerator at 4°C for nanoindentation and SEM.

Nanoindentation

All mandibular left incisors after being ground transversely were used for nanoindentation measurements. In this study, the Nano-indenter XP (MTS Systems, Oak Ridge, TN) was used at room temperature. The system had force and displacement resolution of 50 nN and 0.01 nm; the rate and indentation depth were limited to 10 nm/second and 800 nm, respectively. At least 25 indentations were impressed on the middle region of mature enamel per incisor. The hardness and modulus can be determined from the load-displacement curves, as documented by Oliver-Pharr [13]. Only the data from the indentations with their entire impressions in a single prism region (nearly 15 indentations per incisor, 60 per strain) were usable; others impressed in the sheath or interprism were left out of statistical study. Finally, TG results were compared with WT results by *t*-test at the 95% confidence level.

SEM Analysis

All mandibular and maxillary right incisors were cut and ground longitudinally and transversely (Fig. 1), then lightly etched with 1% nitric acid for 60 seconds. After acid etching, enamel rods can be distinguished from interprisms and sheaths in SEM by a difference in the distinctness of crystal orientation. The samples were dehydrated through graded ethanol concentrations and exchanged twice in isoamyl acetate with 15 minutes each and then critical point-dried with the Hitachi (Tokyo, Japan) HCP-2. Transverse and longitudinal sections were coated with a layer of gold about 10 nm thick for SEM observation at 10 kV (LEO-1530; Leo Elektronenmikroskopie, Oberkochen, Germany).

FTIRM Analysis

Approximately 10 mg of powders were collected using a rotating drill (RDM-1301D2; DMART, P. R. China) in the mature enamel region of each maxillary left incisor (Fig. 1c). These samples were ground and examined directly on a MAGNA-IR 750 FTIRM (Spectrum, Macclesfield, UK).

Results

In the present study, we compared the differences in enamel mineralization characteristics between the incisors of TG and WT mice from macroscale to nanoscale. In particular, enamel distribution, microstructures, crystal properties, and nanomechanical properties were investigated in detail.

Figure 2 gives a representative example of the SEM morphologies of the transverse sections from WT and TG maxillary right incisors, indicating the aberrations of enamel distribution in TG mice. Firstly, the TG incisors were abnormally wider than WT samples, with around 1 mm width from the mesial to the lateral region (as illustrated in Fig. 1c), which is typical and widespread in other sections. Secondly, the TG enamel contour was not as flat and smooth as WT, especially with many turning points in the fluctuant dentine–enamel junction (DEJ) or enamel surface (arrowheads in Fig. 2b). High magnifications of the turning points are shown in Fig. 2c–f. Moreover, the TG enamel was much thinner than WT and varied from region to region. It should also be clearly noted that the enamel surface was not consecutive everywhere, being interrupted at some regions by exposure of the dentine outside directly (asterisks in Fig. 2b, d).

Figure 3 reveals the prism organization and crystal arrangement patterns of TG compared with WT inner enamel in longitudinal sections of maxillary right incisors. Although the prisms (P1 and P2 in Fig. 3d) could be identified for the different orientations of their component crystals, they were not arranged in single-layered rows of lamellar structure. The TG prism layers followed distorted routes and showed no typical prism decussation organization, which was universally typical in WT inner enamel (Fig. 3a). More interestingly, prisms with crystals perpendicular to the cutting directions (P2) were much fewer than the parallel ones (P1). Beyond that, the interprism between the neighboring prisms was difficult to identify. The absence of these prisms (P2) and interprisms resulted in the loosely packing prism bundles with small holes in TG enamel (asterisks in Fig. 3b), while no such loose spaces were observed in WT enamel. In addition, the crystals among each prism were not arranged as close as those in WT. Also, small gaps or holes could be easily observed between them (asterisks in Fig. 3d).

Several kinds of aberrant crystal arrangement patterns found in the longitudinal sections of TG mandibular right incisors were observed (Fig. 4). The crystals in these regions were not organized into prisms or interprisms, and these aberrations in the crystal pattern could be detected in both mature and immature TG enamel. In some inner enamel regions, as shown in Fig. 4a, b, the crystals did not show a rod-like shape but instead were sheet-like and about 40–50 nm thick. These numerous sheets aggregated parallel with each other to form a “layered pattern” (Fig. 4b). In addition, a “parallel rod-like pattern” was observed in some other regions, in both inner and outer enamel. The rod-like crystals here assembled parallel with each other and formed a uniform structure of micrometer size (Fig. 4d), while at the higher level of organization, these crystals underwent splitting, leaving numerous microcracks between them (asterisks in Fig. 4c, d). More than that, in some inner domains near the DEJ, many of these parallel-parking crystals were found to aggregate tightly into clusters of diameter about 200–500 nm (Fig. 4f). Many such crystal clusters were arrayed loosely together to form a “grass-like pattern” (Fig. 4e, f).

The region of the DEJ was usually vivid and clearly separated the enamel and dentine, while in TG incisors the enamel component clearly protruded into the dentin area abruptly in some regions of the DEJ (Fig. 5a). The crystals in this “extra enamel” were not rod-like but presented a plate-like shape, similar to the morphologies in Fig. 4b. These crystals were

nearly 50–60 nm thick, arranged irregularly, and organized into much looser and more porous enamel (Fig. 5b).

Besides the microstructures of enamel, crystal properties were also investigated in our study by FTIRM. Figure 6 represents the typical FTIRM absorbance spectra from the enamel of these two strains. The areas of the phosphate $\nu_1; \nu_3$ band ($900\text{--}1,200\text{ cm}^{-1}$) and $\nu_2\text{CO}_3^{2-}$ band ($850\text{--}900\text{ cm}^{-1}$) have been labeled. Although there were no apparent differences between the two spectra, the ratio of the integrated areas under the $\nu_2\text{CO}_3^{2-}$ and the phosphate $\nu_1; \nu_3$ peaks were distinct. The ratio of the integrated areas ($\text{CO}_3^{2-}/\text{PO}_4^{3-}$) was previously used to evaluate mineral quality and crystallinity [14–16]. Here, the ratio was larger in TG than WT, indicating poorer crystallinity/maturity. Nevertheless, selected areas of electron diffraction from the two samples by high-resolution transmission electron microscopy exhibited no difference, which were indexed as hydroxyapatite phase (data not shown).

As we all know, variations of the biomineralization characteristics, especially the aberrant prism/crystal organization and cracks in TG enamel, must influence the mechanical properties of enamel. In our study, nanoindentation was used to measure the hardness and elastic modulus of enamel. All usable data were used for preliminary statistical analysis. Means and standard deviations of hardness and elastic modulus were calculated, respectively, from these data and are listed in Table 1. TG enamel exhibited approximately 24.6% lower hardness and 12.3% reduced modulus than WT enamel, which indicated that the abnormal biomineralization in TG enamel did have an effect on its mechanical properties.

Discussion

As we know, alteration at the gene level affects cellular activities and protein self-assembly during organogenesis and, thus, results in alterations to upper orders of tissue organization [17]. We simplify our discussion at several artificially separated levels of organization as proposed by Paine et al. [7].

Gene regulation serves to govern which special proteins are expressed and secreted to form an extracellular matrix and controls the crystal organization by protein-to-protein and protein-to-crystallite interactions at the nanoscale. Due to postnatal PPR activation in odontoblastic cells, expression of amelogenin and ameloblastin was delayed and heterogeneous in the ameloblastic layer of TG mice [10]. The essential roles of amelogenin [3, 18–21] and ameloblastin [3, 22, 23] are important for enamel organic extracellular matrix assembly as well as crystal nuclear organization. Amelogenin undergoes self-assembly to form nanospheres located adjacent to the *a* and *b* surfaces of hydroxyapatite crystallites, which inhibit lateral crystal growth in the *a*, *b* axial direction and leave the crystallite elongation along the *c* axis [24]. In the domains of *coll-1*-caPPR incisor with less expression of amelogenin, crystal growth processes were certainly impaired, such as the prevention of premature crystal–crystal fusion and the modulation of crystal morphology and subsequent elongation [25]. In our study, we found that the crystals failed to organize into bundles but fused to form plate-like and sheet-like crystals, as shown in Figs. 4a, b and 5a, b, similar to the amelogenin-deficient mice [21]. At the same time, ameloblastin serves as a regulator of crystallization [22, 26] and part of the linkage between ameloblasts and the extracellular matrix [27], especially as a cell adhesion molecule for the maintenance of ameloblast differentiation [23]. The lower expression of ameloblastin resulted in a lack of full differentiation of TG ameloblastic cells, as detected by *in situ* hybridization previously [10]. In addition, the ameloblastin located in the periphery of enamel prisms would serve to

maintain the boundaries of prisms and limit the prism–interprism continuity [3]. The absence of ameloblastin here may have an influence on enamel prism organization and result in illegible prism and interprism morphological differences, as displayed in Fig. 3b, d.

The enamel organization at the mesoscale is under the control of the ameloblasts. The ameloblasts control the conformation and vector of movement of the secretory Tomes' processes, which govern the final orientations of prism and interprism [7]. In col1-caPPR incisors, the difficulty identifying the interprism may be due to the abnormally altered ameloblastic differentiation. Furthermore, the impaired differentiation and disorganization of ameloblastic cells probably gave rise to the convergence of prisms without decussation, especially the decrease of the prisms perpendicular to the cutting direction (Fig. 3b). Additionally, abnormal ameloblasts, with their movement outward away from the DEJ, might influence the growing crystallites [28] and, thus, form distorted routes of prisms (Fig. 3b).

At the macroscale level, two special zones of enamel, DEJ and the outermost zone of enamel, represent variations at the beginning and end of enamel development. Teeth develop as a consequence of an elaborate dance of instructive signaling exchanged between odontoblasts and ameloblasts through the DEJ [7]. There is growing evidence indicating that this exchange of biologically relevant signals continues into the mineralization period [29, 30]. In this case, some proteins may be exchanged from the ameloblasts to the odontoblasts, perhaps contributing a development signal that may herald the generation of the DEJ. In TG enamel, the DEJ presented rougher and discontinuous features and even protruded into dentin. One hypothesis is that the excess proteins secreted by those altered ameloblasts induce enamel biomineralization in dentin and synthesize these extra enamel regions [31]. Besides that, the nude dentin without the cover of enamel shell, as shown in Fig. 2b, d, may be due to the absence of ameloblast cells and the lack of related extracellular matrix proteins there. The disorganization of the ameloblast cells at the protective stage may result in turning points (Fig. 2b–f) in the outer enamel surface.

The mechanical properties of mature enamel reflect the organization of its constituent components. The interconnections between prism and interprism and complex cleavage planes limit critical crack size and uncontrolled propagation that would otherwise lead to premature fracture [32]. This prism decussation pattern of crystal organization is helpful for increasing hardness and reducing wear. Here, in col1-caPPR incisor enamel, the disorganized ameloblastic differentiation as well as the heterogeneous expression of amelogenin and ameloblastin resulted in poorer crystallinity/maturity, a disordered crystal arrangement, an increase in porosity, consistently disorganized prism distribution, and destruction of the entire and regular enamel structure, all of which made the mechanical properties decrease dramatically.

The alteration of enamel mineralization and the brittleness are both common features of some tooth diseases. Amelogenesis imperfecta and other diseases are characterized by an abnormal extracellular matrix, abnormal deposition, and abnormal organization of minerals [18, 21]. Here, we observed abnormal crystal organization and enamel distribution in col1-caPPR incisor enamel. Therefore, the study may be helpful for understanding the mechanisms of and therapeutic strategies for these diseases. Further studies are needed to identify the altered protein matrix in abnormal domains. More details are also needed to explain how the altered gene and proteins mediate the mineralization process, affecting the enamel structure during tooth development. Investigations of the alterations in molars and dentine are under way in our laboratory.

Conclusion

Disturbed enamel biomineralization in *coll1-caPPR* incisors was investigated for the first time in our study. We examined the enamel distribution, microstructures, and mechanical properties by SEM, FTIRM, and nanoindentation. The SEM investigations displayed abnormal enamel distribution and altered crystal arrangement in TG incisor, especially the prism organization without decussation and the loosely packing crystals, which were significantly different from WT incisor. FTIRM analysis revealed poorer crystallinity/maturity in TG enamel compared with WT enamel. The loosely packing crystals and abnormal prism organization resulted in inferior mechanical properties in TG enamel. All these differences indicated alterations of the biomineralization process during incisor enamel development in *coll1-caPPR* mice.

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Fig. 1. Schematic representation of a maxillary and mandibular incisor (**a**), its transverse (**b**), and longitudinal (**c**) sections. *Double-headed arrows* show the position of transverse sections. The width was measured from the lateral to the mesial direction. E, enamel; D, dentin; P, pulp. *Asterisk* points to the position of powders for FTIRM

Fig. 2.

Transverse sections of WT (**a**) and TG (**b–f**) maxillary right incisors. E, enamel; D, dentin; DEJ, dentine–enamel junction; L, lateral; M, mesial. TG enamel was different from WT with respect to its uneven thickness and unsmooth DEJ. In addition, the prism decussation was difficult to identify in the outer enamel. *Arrowheads* point to the turning points or furrows on the DEJ and the enamel surface; *asterisks* point to the discontinuity of enamel

Fig. 3. SEM images of longitudinal sections show the inner enamel organization for WT (**a, c**) and TG (**b, d**) maxillary right incisors. P1, P2, prism; IP, interprism. In WT inner enamel, prism decussation is the universal typical structure. The crystals tend to align lengthways and lie parallel to build up into the prism, and the prisms are arranged parallel with each other in single-layered lamellae (rows), separated by interprisms. In TG inner enamel, it was difficult to distinguish the prism and interprism, on account of their looser and distorted arrangement of crystals. *Asterisks* point to the holes between adjacent or decussating prisms in **b** and gaps among a prism in **d**

Fig. 4.

Several different types of aberrant crystal organization patterns were encountered in longitudinal sections of mandibular right incisor enamel. **a, b** “Layered pattern,” constituted by sheet-like crystals. **c, d** “Parallel rod-like pattern,” composed of nearly parallel crystals with numerous cracks. **e, f** “Grass-like pattern,” made up of nearly parallel crystals full of gaps. Note the increased porosity and cracks (*asterisks*) in these patterns

Fig. 5. SEM images characterize the protruding enamel domain, which is surrounded by dentine. It was observed in longitudinal sections of maxillary right incisors. **b** Higher magnification of inner enamel (see regions marked with *asterisk* in **a**)

Fig. 6.
 Typical FTIRM absorbance spectra of enamel from WT and TG incisors. Areas of interest to the present study have been appropriately marked

Table 1

Hardness and elastic modulus characterized by nanoindentation

	Hardness (GPa)*	Elastic modulus (GPa)*
Transgenic	57.45 ± 2.10	6.89 ± 0.30
Wild-type	76.16 ± 3.50	7.86 ± 0.56

* $P < 0.001$ (t -test)