Published in final edited form as:

Connect Tissue Res. 2014 August ; 55(0 1): 38–42. doi:10.3109/03008207.2014.923871.

Correlation of ameloblastin with enamel mineral content

John D. Teepe¹, James E. Schmitz², Yuanyuan Hu³, Yoshihiko Yamada⁴, Roberto J. Fajardo², Charles E. Smith^{3,5}, and Yong-Hee P. Chun⁶

¹Department of Periodontics, Dunn Dental Clinic, Lackland Air Force Base, San Antonio, TX, USA

²Department of Orthopaedics RAYO, Carlisle Center for Bone and Mineral Imaging, School of Medicine, University of Texas Health Science Center, San Antonio, TX, USA

³Department of Biologic and Materials Sciences, University of Michigan, Dental Research Lab, Ann Arbor, MI, USA

⁴Laboratory of Cell and Developmental Biology, National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD, USA

⁵Department of Anatomy & Cell Biology, McGill University, Montreal, Quebec, Canada

⁶Department of Periodontics, Dental School, University of Texas Health Science Center, San Antonio, TX, USA

Abstract

In enamel formation, the deposition of minerals as crystallites starts when the mineralization front first forms at the start of the secretory stage. During maturation, the enamel layer accumulates significant amounts of new mineral as the crystallites grow in volume. Inversely related to mineral gain is loss of protein and water from the forming enamel. Both ameloblastin (Ambn) and enamelin are essential components for formation of a functional enamel layer. The aim of this study was to quantify the proportion of mineral and non-mineral material present in developing enamel relative to Ambn concentration using Ambn mutant mice mated with others overexpressing full-length Ambn from the mouse amelogenin promoter at lower (+), similar (++) or higher (+++) concentration than normal. Mandibular incisors (age: 7 weeks, $n=8$) were imaged by micro-computed tomography and the enamel was analyzed from the apical region to the incisal edge in sequential 1.0 mm volumes of interest. Mineral density was determined using a series of hydroxyapatite (HA) phantoms to calibrate enamel density measurements. At the site where the mandibular incisor emerged into the oral cavity, the enamel volume, mineral weight, and mineral

© 2014 Informa Healthcare USA, Inc

Correspondence: Yong-Hee P. Chun, DDS, MS, PhD, Assistant Professor, Department of Periodontics, Dental School, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78229, USA. Tel: 210-567 3520. Fax: 210-567 6858. chunyh@uthscsa.edu.

The authors have no conflicts of interest related to this study.

Supplementary material available online

Supplementary Tables 1 and 2 (contained in one document)

Supplementary Table 1: Volume per enamel segment

Supplementary Table 2: % Mineral and % non-mineral per enamel segment

Supplemental material available at informahealthcare.com/cts

density were reduced when Tg Ambn was expressed at lower or higher levels than normal. While in wild-type the % mineral was >95%, it was negligible in Ambn^{-/-}, 22.3% in Ambn^{-/-}, Tg(+), 75.4% in Ambn^{-/-}, Tg(++), and 45.2% in Ambn^{-/-}, Tg(+++). These results document that the deposition of mineral and removal of non-mineral components are both very sensitive to expressed Ambn concentrations.

Keywords

Ameloblastin; enamel; enamel biomineralization/formation; mineralized tissue/development; micro-computed tomography; mineralization front

Introduction

Enamel mineralizes into the hardest biomineral in a two-stage process. At the onset of secretion of enamel proteins from the apical pole of ameloblasts during the secretory stage, mineral ribbons are organized via the Tome's processes of these cells to form rods (prisms) that span the enamel layer from the dentino-enamel junction (DEJ) to just beneath the outermost enamel surface (1). This secretory enamel has initially a low mineral content of about 15% and reaches about 30% when it transitions into the maturation stage (2). During the maturation stage, enamel matures and reaches 95% mineral (by weight and volume) in alternating cycles of removal of enzymatically degraded enamel proteins and accumulation of hydroxyapatite (HA) (2).

Secretory stage enamel mineralization is assisted by the non-mineral components of amelogenin and nonamelogenin proteins. Although the non-amelogenin protein ameloblastin (Ambn) accounts for only small amounts of total expressed enamel proteins, it is essential for appositional growth of enamel and to maintain the mineralization front during the secretory stage. When Ambn is mutated in mice to have exons 5 and 6 replaced with a PGK neo cassette (3), no enamel is formed. Instead, teeth are covered by a thin, dysplastic mineralized layer (4, 5). The functional importance of Ambn for enamel formation was demonstrated by reconstituting full-length Ambn in Ambn mutant mice which results in recovery of the enamel thickness (6).

The purpose of this study was to determine by microcomputed tomography (μ CT) the relevance of Ambn concentration on the volume of the enamel layer, the % mineral/volume and % non-mineral/volume, when lower, equal or higher than wild-type levels of Ambn are expressed in Ambn mutant mice.

Methods

Protocol approval and animals

The IACUC of the University of Texas Health Science Center at San Antonio approved protocols for the use and handling of animals. Mice were housed in a facility with a 12-h light-dark schedule with unrestricted access to food and drink. Animals were provided with a hard diet (7012 Teklad LM-485, Harlan Laboratories, Indianapolis, IN) and a soft diet (DietGel[®] Recovery, Clear H₂O[®], Portland, ME). The water was treated by reverse osmosis.

Transgenic Ambn was overexpressed in separate mouse lines in three different concentrations (6). Transgenic Ambn expression was either below wild-type (Tg+), similar to wildtype (Tg++) or higher than wild-type (Tg+++) as determined by Western Blotting in developing molar enamel (6). Mice expressing transgenic Ambn were cross-bred with mice lacking Ambn exons 5 and 6 (Ambn mutant) (3, 4). Genotype of animals was identified by PCR using DNA from tail snips. All mice were bred within the C57BL/6 background. At 7 weeks of age, animals were intracardially perfused with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) with 0.2% glutaraldehyde, pH 7.4. Hemi-mandibles from eight animals per genotype were dissected and stored at 4 °C in PBS containing 0.2% PFA, pH 7.4.

Micro-computed tomography

Hemi-mandibles were positioned vertically into a tube with 70% ethanol and aligned to the occlusal plane. The scanning protocols were performed as described earlier (7) in a desktop scanner (Bruker SkyScan 1172, Kontich, Belgium) with a 0.5-mm aluminum filter at 60 kV, 167 mA beam intensity and a 0.35° rotation step with 1090 ms exposure time at each step. The image pixel size was 5.0 mm. The samples were reconstructed using NRecon (Bruker SkyScan, Aartselaar, Belgium) using a polynomial correction algorithm.

Enamel formation can be continuously monitored in rodent incisors by using microdissected strips (8) or segments created by μ CT (7). One millimeter volumes of interest (VOI) were determined along the length of the incisor, beginning distal to the third molar (Figure 1). The VOI boundaries were manually drawn along the DEJ and surface of the enamel. Enamel volume and enamel mineral density were measured in these VOI. A series of HA phantoms with the densities of 0.25, 0.75, and 2.93 g/cm³ were used to calibrate the enamel density measurements. μ CT-based enamel density measurements were compared and validated against density measurements determined by ashing (mineral weight) (8) and electron microscopy (enamel volume) (7).

The % mineral and % non-mineral components were calculated in reference to the expected mineral weight estimated from cross-sectioned wild-type mandibular incisors in 1-mm tall VOI (7).

Statistical analyses

Data were recorded in Microsoft Office Excel software. Means and standard deviations were calculated in Excel and GraphPad InStat (La Jolla, CA). Volume, % mineral and % non-mineral of the Ambn rescue genotypes were compared using analysis of variance with a significance level set at $p = 0.05$. Data were tested for normality and post-hoc testing was done with the Tukey–Kramer method.

Results

In Ambn+/+ mice, incisor enamel mineralized gradually from the apical loop to the gingival margin and formed a homogenous layer (Figure 1). Similarly, Ambn+/- mice had an enamel layer with qualities comparable to wild-type. In contrast, Ambn mutant (-/-) animals lacked true enamel; instead a thin crust, and ectopic mineralizations were evident covering the

dentin and other ectopic mineralizations were seen within the enamel organ space itself. The reconstitution of Ambn in Ambn^{-/-} animals with Tg(++) resulted in an enamel layer most similar to wild-type with gradual build-up of enamel thickness and homogenous radiodensity. When Ambn was expressed at lower concentration Tg(+), the appositional growth and mineralization of enamel were delayed and reduced throughout enamel formation. A high level of Ambn expression Tg(+++) in Ambn^{-/-} animals resulted in heterogeneously mineralized enamel with a nodulated enamel surface and ectopic mineralizations.

Enamel volume in 1-mm increments increased up to segment 4 in wild-type animals and decreased slightly in volume throughout the maturation stage reaching $0.0648 \pm 0.0057 \text{ mm}^3$ at eruption (Figure 2A). Enamel volume in Ambn^{+/-} mice was not different from wild-type (Supplementary Table 1). The mineralized material covering the incisors from Ambn mutant (^{-/-}) mice occupied a minute volume of $0.0263 \pm 0.0058 \text{ mm}^3$. Enamel volumes were not statistically different in Ambn^{-/-} mice mated with Tg(+) or Tg(+++) expressing mice compared to Ambn mutant mice alone. When Ambn was expressed at medium concentration (Tg++), enamel volume was $0.0544 \pm 0.0052 \text{ mm}^3$, which differed from Ambn mutant, Ambn^{-/-} mice with Tg(+) or Tg(+++), as well as from wild-type enamel volumes.

Mineral within the enamel layer was gained rapidly from segment 3 through 8 in wild-type and Ambn^{+/-} animals (Figure 2B). In wild-type animals, the mineral increased from 26.4 to 95.1% per volume. Throughout the length of incisors in Ambn mutant mice, the abnormal material covering the dentin was statistically different at around 5.1% mineral by volume. During what is normally early maturation stage by distance along the length of the incisor (segment 3), the %mineral in forming enamel of Ambn^{-/-} animals expressing Tg(+) or Tg(+++) was not different from Ambn mutant mice. During maturation stage both Tg lines gained mineral and reached statistical difference from Ambn mutants but remained statistical different from wild-type mice (had less mineral). When Ambn was expressed transgenically at medium concentration (Tg ++), the mineral in enamel did not differ initially from wild-type, but in late maturation stage it was only 75.4% mineral by volume.

Inverse to the accumulation of mineral, the non-mineral components decreased from 73.6% to 4.9% per enamel volume (Figure 2C) in wild-type and Ambn^{+/-} mice. In Ambn^{-/-} animals, the non-mineral content did not change much across the length of the incisors (Figure 2C). In Ambn Tg(+) and (++) animals, only small amounts of non-minerals were removed to 77.7% and 54.8% by volume in late maturation stage (Figure 2C, segment 8). Among the Ambn Tg reconstituted mice, the medium expressers (Tg++) had the greatest reduction in non-minerals from 72.1 to 24.6% and reached statistical difference from Ambn mutants and from wild-type mice (Figure 2C).

Discussion

The reconstitution of Ambn mutant mice with transgenic Ambn resulted in partial recovery of enamel volume, % mineral and % non-mineral when the Ambn concentration was similar to wild-type. Appropriate removal of nonminerals and accumulation of mineral failed in animals expressing transgenic Ambn in the mutant background. The more the concentration

of transgenic Ambn deviated from wild-type, as in Tg(+) and Tg(+++) expressing animals, the more severely the enamel volume, removal of non-mineral components and gain in mineral were compromised. The mineralization front adjacent to the ameloblast membrane is sensitive for the concentration of Ambn needed to deposit an enamel layer and to form mineral ribbons in vivo. In the absence of functional Ambn as in Ambn mutant mice, the mineralization front along the ameloblast membrane is missing in secretory stage and mineral ribbons fail to initiate and extend, thus no enamel is formed. When the Ambn concentration is too low (Tg+), appositional growth of enamel is suppressed. When the Ambn concentration exceeds normal (Tg+++), some appositional growth occurs but non-minerals are not sufficiently removed from the matrix, thereby blocking growth of crystallites in length and width.

Transgenic Ambn was capable of partially reconstituting the mineralization front, gaining appositional growth of enamel and clearance of enamel proteins from the matrix, but only when the concentration was close to wild-type. Besides Ambn, the non-amelogenin protein enamelin is essential for the mineralization front (9). Both non-amelogenins are expressed in distinctly lower concentrations of 3–5% compared to amelogenin at 90%. The precise importance of this disproportionate expression and the molecular mechanisms to maintain the mineralization front in functional condition are unclear but obviously very important.

The Ambn mutant mice express a protein having 279 residues that is secreted into the matrix and efficiently degraded (3). Beyond failure in the mineralization front it is not clear if this mutant protein could exert some dominant negative effects or alter its physiologic function. Interestingly, heterozygous mice expressing mutant Ambn and wild-type Ambn in equal amounts do not display an enamel phenotype. A mouse model without a secreted, residual Ambn protein is required to determine the contribution of mutant Ambn protein to the mutant and reconstituted phenotypes.

Conclusion

Enamel hypoplasia and hypomineralization in Ambn mutant mice can be partially reversed when Ambn is reconstituted transgenically at near normal concentrations. The appositional growth of mineral and removal of non-mineral components are both very sensitive to expressed Ambn concentrations. Ambn concentration appears to control the level at which enamel will ultimately mineralize through of direct, indirect, or combined pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

μCT image data were acquired at RAYO, The Carlisle Center for Bone and Mineral Imaging in the UTHSCSA Department of Orthopaedics.

Declaration of interest

This research was funded by the National Institutes of Health, Bethesda, K08 DE022800 (YPC), S10 RR025687 (RJF), the UTHSCSA Department of Periodontics and the UTHSCSA program for Research Core Laboratories.

References

1. Simmer JP, Papagerakis P, Smith CE, Fisher DC, Rountrey AN, Zheng L, Hu JC-C. Regulation of dental enamel shape and hardness. *J Dent Res.* 2010; 89:1024–1038. [PubMed: 20675598]
2. Smith CE. Cellular and chemical events during enamel maturation. *Crit Rev Oral Biol Med.* 1998; 9:128–161. [PubMed: 9603233]
3. Wazen R, Moffatt P, Zalzal SF, Yamada Y, Nanci A. A mouse model expressing a truncated form of ameloblastin exhibits dental and junctional epithelium defects. *Matrix Biol.* 2009; 28:292–303. [PubMed: 19375505]
4. Fukumoto S, Kiba T, Hall B, Iehara N, Nakamura T, Longenecker G, Krebsbach PH, Nanci A, Kulkarni AB, Yamada Y. Ameloblastin is a cell adhesion molecule required for maintaining the differentiation state of ameloblasts. *J Cell Biol.* 2004; 167:973–983. [PubMed: 15583034]
5. Smith CE, Wazen R, Hu Y, Zalzal SF, Nanci A, Simmer JP, Hu JC-C. Consequences for enamel development and mineralization resulting from loss of function of ameloblastin or enamelin. *Eur J Oral Sci.* 2009; 117:485–497. [PubMed: 19758243]
6. Chun Y-HP, Lu Y, Hu Y, Krebsbach P, Yamada Y, Hu JC-C, Simmer JP. Transgenic rescue of enamel phenotype in *Ambn* null mice. *J Dent Res.* 2010; 89:1414–1420. [PubMed: 20940352]
7. Schmitz JE, Teepe JD, Hu Y, Smith CE, Fajardo RJ, Chun Y-HP. Estimating mineral changes in enamel formation by ashing/BSE and MicroCT. *J Dent Res.* 2014; 93:256–262. [PubMed: 24470541]
8. Smith CE, Nanci A. A method for sampling the stages of amelogenesis on mandibular rat incisors using the molars as a reference for dissection. *Anat Rec.* 1989; 225:257–266. [PubMed: 2683870]
9. Simmer JP, Richardson AS, Hu Y-Y, Smith CE, Hu JC-C. A postclassical theory of enamel biomineralization. . . and why we need one. *Int J Oral Sci.* 2012; 4:129–134. [PubMed: 22996272]

Figure 1.

Sagittal μ CT sections displaying the enamel from apical loop to the incisal tip of $Ambn$ mutant mice rescued with transgenic $Ambn$. Arrow indicates the starting point for the volume of interest (VOI). Arrowheads point to ectopic mineralizations. All mice were 7 weeks old.

Figure 2.

Enamel volume, % mineral and % non-mineral components by volume in Ambn mutant mice rescued with transgenic Ambn. In mandibular incisor enamel, the earliest radiodense signal with μ CT analysis is detected at early maturation stage (segment 3). The enamel volume was determined for wild-type, Ambn mutant and Ambn mutant rescued with transgenic Ambn (A). For statistical significances, see Supplementary Table 1. % mineral

(B) and % non-mineral (C) components are inverse of each other. For statistical significances, see Supplemental Table 2. All mice were 7 weeks old.