Enamelysin (Matrix Metalloproteinase 20)-deficient Mice Display an Amelogenesis Imperfecta Phenotype*

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Enamelysin is a tooth-specific matrix metalloproteinase that is expressed during the early through middle stages of enamel development. The enamel matrix proteins amelogenin, ameloblastin, and enamelin are also expressed during this same approximate developmental time period, suggesting that enamelysin may play a role in their hydrolysis. In support of this interpretation, recombinant enamelysin was previously demonstrated to cleave recombinant amelogenin at virtually all of the precise cleavage sites known to occur in vivo. Thus, enamelysin is likely an important amelogenin-processing enzyme. To characterize the in vivo biological role of enamelysin during tooth development, we generated an enamelysin-deficient mouse by gene targeting. Although mice heterozygous for the mutation have no apparent phenotype, the enamelysin null mouse has a severe and profound tooth phenotype. Specifically, the null mouse does not process amelogenin properly, possesses an altered enamel matrix and rod pattern, has hypoplastic enamel that delaminates from the dentin, and has a deteriorating enamel organ morphology as development progresses. Our findings demonstrate that enamelysin activity is essential for proper enamel development.

Dental enamel covers the crown of the tooth and is unique among mineralized tissues because of its high mineral content, large crystals, and organized prism pattern. Other mineralized tissues such as bone, dentin, and cementum are composed of ~20% organic material. In contrast, mature enamel has less than 1% organic matter by weight (1, 2). Moreover, enamel crystallites possess a volume that is 100 times greater than the volume of crystallites found in other mineralizing tissues. These enamel crystallites form enamel rods that, in turn, form a unique interlacing (decussating) prism pattern. As a result, dental enamel is the hardest substance in the body. Its hardness is intermediate between that of iron and carbon steel, yet it also has a high elasticity (3).

Although mature enamel is a very hard protein-free tissue, it does not start this way. Enamel development (amelogenesis) consists of several stages that include the secretory, transition, and maturation stages. During the secretory stage, enamel crystallites elongate into long thin ribbons that are only a few nanometers thick (about 10 nm) with a width of ~30 nm (4, 5). The ribbons are evenly spaced, are oriented parallel to each other, and grow in length but very little in width and thickness. Ultimately, enamel crystal length determines the final thickness of the enamel layer as a whole (for review, see Ref. 6). It is during the secretory stage that the columnar-shaped ameloblast cells, located adjacent to the forming enamel, secrete specialized enamel proteins into the enamel matrix. These proteins include amelogenin (7), ameloblastin (8), and enamelin (9). Amelogenin is the predominant component and comprises ~90% of total enamel matrix protein (10). Interestingly, the full-length enamel proteins are found only at the mineralizing front, suggesting that they participate in crystal elongation (11–19). In contrast, the protein cleavage products are found throughout the enamel layer, suggesting that they prevent crystallite growth in width and thickness (16).

Enamelysin is a member of the matrix metalloproteinase family, and its mRNA has been cloned from pig (20), human (21), cow (22), and mouse (23). Enamelysin is secreted into the enamel matrix during the secretory stage through transition stage of enamel development (24–27). Because enamelysin is present in the mineralizing front, it is thought to participate in the cleavage events that allow the crystals to grow in length but not in width or thickness (25). Previously, recombinant enamelysin was demonstrated to cleave recombinant amelogenin at virtually all of the precise cleavage sites that were demonstrated to occur in vivo (28). Thus, enamelysin was identified as a predominant amelogenin-processing enzyme.

As the secretory stage ends and the transition stage begins, the ameloblasts shrink in size and down-regulate protein release into the enamel matrix. These changes are associated with an end of the elongation of enamel crystals. The transition stage is followed by the maturation stage, where enamelysin expression is eliminated, and the crystallites grow in width and thickness but no longer in length. The remaining proteins within the enamel matrix are degraded by an enamel matrix serine proteinase (Kallikrein-4) before their export out of the enamel (25, 29–32). Enamel attains its final hardened form at the completion of the maturation stage. These general features of amelogenesis are remarkably consistent among different species (33).

Enamelysin is unique among the MMP family members because of its highly restricted pattern of expression. One study...
Enamelysin (MMP-20) Knockout Mice

assessed 51 different cell lines for enamelysin expression, but none were positive (34). Conversely, enamelysin expression was observed in pathologic tissues such as in ghost cells of calcifying odontogenic cysts (35), odontogenic tumors (36), and human tongue carcinoma cells (37). Recently, enamelysin expression was also observed in bradykinin-treated granulosa cells isolated from the follicles of porcine ovaries (38). However, with the exception of the ameloblasts of the dental papilla (20), none were positive (34). Conversely, enamelysin expression was performed at a constant current of 20 mA per gel for ~7 h. After electrophoresis, protein gels were silver-stained (Amersham Biosciences), and zymography gels were washed twice for 10 min in 50 ml of 2.5% Triton X-100 solution (2.5% Triton X-100 in 100 mM Tris-HCl buffer [pH 8.0]). The gels were incubated for 1–2 days at 37 °C in 50 ml Tris-HCl buffer (pH 7.2) containing 10 mM CaCl2 and stained with Coomassie Brilliant Blue (CBB) R-250 solution (0.2% CBB R-250, 5.8% acetic acid, and 30% methanol) for 20 min and destained with 10% methanol and 10% acetic acid until clear bands of substrate lysis were observed (41).

**Histology and Scanning Electron Microscopy**—Incisors obtained from three euthanized wild type, three heterozygous, and six enamelysin null mice were fixed in 5% neutral formalin/saline overnight, incubated in phosphate-buffered saline containing 0.1% Triton X-100 for 8 h, rinsed overnight with running water, and decalcified in 20% sodium citrate, 45% formic acid for 2 weeks. This and all subsequent incubations were performed at ambient temperature. The jaws were dehydrated in a graded series of ethanol washes and embedded in paraffin for sectioning. Deparaffinized and rehydrated sections were stained with hematoxylin/eosin. For scanning electron microscopy, eroded molar and incisor teeth were either examined whole or fractured transversely, air-dried, fastened to stubs, sputter-coated, and examined using a JEOL 6400 scanning electron microscope.

**RESULTS**

**Targeted Disruption of the Enamelysin Locus**—The mouse enamelysin gene includes 10 exons and is located within the MMP cluster at the centromeric end of chromosome 9 (42). To disrupt the functional expression of the enamelysin gene, a 10.6-kb-segment-containing sequence starting at the 3′ end of exon 2 and extending through most of intron 5 was modified such that the majority of intron 4 and exon 5 was replaced by a phosphoglycerate kinase promoter-controlled hypoxanthine-guanine phosphoribosyltransferase minigene (Fig. 1A) (39, 43). Exon 5 encodes the highly conserved zinc-binding site (HEXGHXXGXXH) present in the catalytic domains of the MMP family. This deletion renders any polypeptide expressed from this mutant gene catalytically inactive. The targeting construct was transfected by electroporation into HM-1 (hypoxanthine-guanine phosphoribosyltransferase-deficient mouse embryonic stem) cells (40), and hypoxanthine/thymine/aminopterin-resistant clones were selected for further characterization. Targeted alleles were identified by PCR and confirmed by Southern blot analysis. Chimeric offspring derived from two individual cell clones were mated to C57Bl/6 mice, and germ line transmission was obtained with chimeras from both clones. Interbreeding of heterozygous mice yielded the expected Mendelian distribution of homozygous mutant (enamelysin−/−), heterozygous (enamelysin+/-), and wild type (enamelysin+/+) mice (Fig. 1B). Total RNA prepared from enamelysin−/−-homogenized incisors probed with an exon 5-specific probe demonstrated the absence of transcripts containing this exon (Fig. 1C). Zymography of proteins extracted from 4.0–4.5-day-old first molars verified the absence of enamelysin activity in the enamelysin-deficient mice (Fig. 1C). Note that two enamelysin bands are present on the zymogram. A study in which native enamelysin was purified from porcine enamel suggests that the two bands represent active intact enamelysin and active...
FIG. 1. Generation of enamelysin knockout mice. A, a map of the targeted phosphoglycerate kinase-hypoxanthine-guanine phosphoribosyltransferase minigene demonstrating the loss of most of intron 4 and exon 5. Exons are depicted as dark boxes. Indicated below is the change in EcoRI restriction pattern between the wild type and targeted enamelysin gene. B, PCR and Southern analysis of the F2 generation mice. Primers p03-p06 were used for PCR analysis where the 5' primers were specific for intron 4 (wild type (wt)) or the hypoxanthine-guanine phosphoribosyltransferase minigene (null). Southern analysis was performed with an exon 6-specific probe after an EcoRI restriction digest of genomic DNA. A 7.3-kb band demonstrated the presence of the wild type allele, and a 6.5-kb band demonstrated the presence of the knockout (k/o) allele. C, total RNA from incisors was probed with an exon 5-specific probe to confirm the loss of exon 5 in the homozygous null mice. Proteins from immature mineralizing molars were subjected to zymography to demonstrate the absence of enamelysin activity in the null mice. Note the doublet present at ~42–46 kDa is missing in the null molars. This doublet represents zones of casein degradation by enamelysin proteins (26) that differ in the size of their hemopexin domains (M, marker; null, enamelysin knockout).
enamelysin with at least one cut site present within its hemopexin domain.

Characterization of Null Mouse Enamel—Maxillas from wild type and enamelysin null mice were removed, the periradicular bone was dissected away, and the exposed molars were prepared for scanning electron microscopy. The first maxillary molars from a wild type (Fig. 2A) and enamelysin null mouse are shown (Fig. 2B). The dashed lines in Fig. 2 encompass the enamel-free areas of each molar. As shown in the wild type, enamel-free areas are normally present in the mouse molar at the marsal plateau of the cusps. These areas provide troughs that are necessary for the efficient side-to-side grinding of ingested food. In the enamelysin null mouse, however, the enamel that surrounds the cusps is virtually absent. Only the cervical margin of the tooth had an enamel covering that remained (Fig. 2). Thus, enamel from the null mouse delaminates from the dentin surface.

To determine whether the characteristic decussating rod pattern was altered in enamel from enamelysin null mice, incisors were fractured and prepared for scanning electron microscopy. The fracture plane of the wild type and heterozygous tooth extended through the enamel and dentin (Fig. 3, A and B), whereas in the null mouse, fracture planes of enamel and dentin were separate (Fig. 3C). This suggests that the null mouse enamel does not adhere properly to the dentin surface.

Littermate wild type and heterozygous mice had an inner enamel layer (100 μm) consisting of alternating rows of enamel rods decussating at about 90°, an outer enamel layer of parallel rods (15 μm) slightly inclined to the tooth surface, and a surface layer without rods (6 μm). Inspection of the littermate null mouse inner enamel rods revealed the complete absence of the typical decussating rod pattern, and enamel rod diameters were notably uneven (Fig. 3C). Fractures in the sagittal plane of null mouse incisors (not shown) did reveal the three distinct enamel layers; that is, the inner enamel layer (50 μm) with parallel rods inclined at about 45° to the dentin surface, an outer enamel layer (15 μm) with rods nearly parallel to the tooth surface, and a surface layer without rods (5 μm). In addition to the abnormal rod pattern present in the null mice, a comparison of the enamel thickness from the dentin/enamel

Fig. 2. Examination of wild type and null mouse molars. Scanning electron micrograph of a first maxillary molar from a wild type (A) and an enamelysin null mouse (B). Dashed lines encircle the enamel-free areas present on each molar. Note the pattern of enamel-free areas that are typical of rodent molars at the marsal plateau of the cusps (A). In contrast, the first molar from the enamelysin knockout mouse contains very little enamel (B). The only enamel that remains is the enamel that surrounds the crown near the gingival margin. Most of the enamel has delaminated from the dentin.

Fig. 3. Examination of littermate enamel prism patterns. Enamel prism pattern of fractured incisors from a wild type (A), heterozygous (B), and enamelysin null (C) mouse. The enamel thickness is ~120 μm in the wild type and heterozygous but is approximately only 70 μm in the null animal. The typical decussating inner enamel rod pattern can be observed in the wild type (A) and heterozygous (B) but is absent in the enamel from the enamelysin null mouse (C). Note the enamel from the null mouse did not fracture in the same plane as the dentin (De), indicating a faulty dentin/enamel junction.

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junction to the enamel surface revealed that the null mice had a significantly thinner (hypoplastic) layer of enamel (70 μm) than did the wild type (120 μm) mice (Fig. 3). Thus, the enamelysin null mouse incisor had enamel that fractured independently of the dentin, abnormal enamel rod pattern, uneven enamel rod diameter, and hypoplastic enamel.

The Enamelysin Null Mouse Tooth Morphology—An advantage of observing tooth development in rodents is that rodent incisors are continuously erupting, and therefore, all the stages of tooth development are present along each forming incisor throughout adult life. A morphological comparison of enamel development present in a demineralized incisor from littermate wild type mice (Fig. 4, A–C), heterozygous mice (Fig. 4, D–F), and enamelysin null mice (Fig. 4, G–I) is shown. Because the tissues were demineralized, only protein is observed. Thus, for the wild type and heterozygous mice (B and E), the matrix was sparse and lightly stained, indicating an increase in mineralization and the loss of protein. In contrast, the enamel matrix protein from the null mouse (H) persisted. In the late maturation stage the enamel matrix of wild type (C) and heterozygous (F) mice was mostly removed. Conversely, enamel matrix in null mice persisted, an abnormally thick layer of protein separated the ameloblasts from the enamel surface (I), and nodule-like formations surrounded by ameloblasts were observed (I, arrows).

Fig. 4. Examination of littermate incisor enamel organ morphology. Demineralized sections of wild type (A–C), heterozygous (D–F), and enamelysin null (G–I) mice showing ameloblasts (Am), enamel space (En), and dentin (De). The wild type and heterozygous sections show tall secretory-stage (A and D) ameloblasts with Tome’s processes penetrating into stained proteins of the enamel layer. The secretory-stage ameloblasts from null mice (G) show ameloblasts that do not have discernible Tome’s processes within the enamel protein layer. In the early maturation stage (B, E, and H) ameloblast length was reduced for all incisors examined. For the wild type and heterozygous mice (B and E), the matrix was sparse and lightly stained, indicating an increase in mineralization and the loss of protein. In contrast, the enamel matrix protein from the null mouse (H) persisted. In the late maturation stage the enamel matrix of wild type (C) and heterozygous (F) mice was mostly removed. Conversely, enamel matrix in null mice persisted, an abnormally thick layer of protein separated the ameloblasts from the enamel surface (I), and nodule-like formations surrounded by ameloblasts were observed (I, arrows).

Fig. 5. Examination of null mouse tooth proteins. Immature molars from 4-day-old mice were dissected free of tissue, extracted for proteins, and subjected to SDS-PAGE. Note that the null mouse has a strong amelogenin band of ~27 kDa, whereas the wild type (Wt) has a very weak band at this position. Also note that several lower Mr amelogenin bands are missing in the null lane compared with the bands present in the wild type lane.
eralized prism structures (Fig. 4, A and B). This organized enamel protein pattern is absent in the enamelysin null mouse (Fig. 4, G and H). Thus, in comparison to wild type and heterozygous animals, the morphology of the enamelysin null mouse incisor displays hypoplastic enamel, ineffective removal of proteins from the enamel matrix, a disorganized protein pattern, and an increasingly disorganized ameloblast morphology as development progresses.

**Enamelysin Null Mouse Display Altered Amelogenin Processing**—To directly demonstrate that enamelysin cleaves amelogenin in vivo, amelogenins were extracted from 4.0–4.5-day-old mouse molars and size-separated by SDS-PAGE. A clearly different pattern of amelogenin degradation was evident between the wild type and null mice (Fig. 5). Amelogenin proteins from the null mice displayed a prominent band at ~27 kDa that was only faintly detectable in the amelogenins from the wild type controls. Only one amelogenin band of less than ~23 kDa was present in the enamel from the null mice, whereas in the controls at least 5 bands were present below this molecular mass. Thus, enamelysin activity is responsible for generating at least four different amelogenin isoforms that are present in normally maturing dental enamel.

**DISCUSSION**

In summary, the enamelysin null mouse does not process amelogenin properly, possesses an altered enamel protein and associated rod pattern, has hypoplastic enamel, has enamel that delaminates from the dentin, and has a deteriorating tooth morphology as enamel development progresses. Previously, several studies show that recombinant enamelysin cleaves recombinant amelogenin (21, 24, 26, 44), including a study demonstrating that recombinant amelogenin was cleaved at virtually all of the precise cleavage sites that had previously been observed in vivo (28). However, until now (Fig. 5), no study has presented direct evidence demonstrating that enamelysin is responsible for these cleavages in vivo. Because enamelysin is expressed primarily during the secretory stage of amelogenesis when the crystals grow in length, it appears that enamelysin functions to initiate hydrolysis of the structural enamel matrix proteins so that the enamel crystals may grow in length. Prevention of this process by the elimination of enamelysin activity results in thin, brittle enamel that does not mature properly. Enamelysin activity is therefore essential for proper enamel development.

In addition, we have observed (not shown) that the first molar of the null mouse possesses less enamel than the second molar, which in turn, has less enamel than the third molar. The mouse molars erupt in this very sequence, from first to third. The same phenomenon was observed in incisor teeth. Intact enamel covered the labial surface of the recently erupted incisor portion near the gingival margin, but at the incisal tip, the enamel was missing. This enamel wear pattern suggests that the teeth erupt with a complete covering of enamel but that over time the malformed enamel wears or chips away presumably due to normal stresses encountered during mastication.

Amelogenin comprises ~90% of the organic component of developing enamel. Thus, the lack of amelogenin processing in the enamelysin null mouse is likely an important aspect of the null mouse phenotype. Previously it was demonstrated that a solitary point mutation (proline to threonine) in exon 6 of the amelogenin gene caused amelogenesis imperfecta (AI) (45). This missense mutation was positioned at P5 relative to a Trp/Leu enamelysin cleavage site and was demonstrated to reduce the efficiency of hydrolysis by 25-fold compared with hydrolysis of the non-mutated peptide (46). Therefore a small change in amelogenin structure can have a profound effect on enamel development. Also, the hydrolysis of enamel proteins can alter their functional properties. Proteolysis of amelogenin reduces both its crystal binding affinity and its solubility (47–51). Thus, the lack of amelogenin processing in the enamelysin null mouse likely eliminated necessary changes in the physical properties of amelogenin that are essential for proper enamel development.

Interestingly, during the late maturation stage, the ameloblasts of the null mouse sometimes surrounded abnormal nodular structures. In addition, an abnormally thick layer of protein appeared to separate the ameloblasts from the enamel surface (Fig. 4f). This result was difficult to interpret given that both enamelysin and amelogenin are not normally expressed during this late stage of enamel development. Perhaps, pre-processing of enamel proteins by enamelysin is necessary for their proper removal from the enamel matrix and/or their subsequent degradation by the ameloblasts.

Because, the dental enamel disease amelogenesis imperfecta affects only dental enamel, the phenotype/genotype of the enamelysin null mice suggest that one form of human AI may be caused by the recessively inherited inactivation of the enamelysin locus. The human amelogenin gene in the p21.1-p22.3 region of the X chromosome and the human enamelin gene at 4q11-q21 are loci in known cases of AI (52, 53). The human enamelysin gene locates to 11q22.3, which has not yet been identified as an AI locus. However, in contrast to the phenotype observed for mutations in the amelogenin gene (X-linked) or the enamelin gene (autosomal-dominant), an enamelysin defect would likely be autosomal-recessive and, therefore, less prevalent within the population. Thus, the likelihood of identifying an enamelysin-deficient AI patient is greatly reduced compared with the known genes that cause AI.

An intriguing aspect of the enamelysin null mouse is that because it displays a severe and profound phenotype and survives to breed, it may be useful for transgenic studies to assess the functional significance of MMP domain structure. MMPs are characterized by a domain structure that consists of a signal peptide of ~20 amino acid residues that is removed after it has directed secretion of the enzyme from the cell, a propeptide composed of ~80 amino acids that folds back to mask and inhibit the catalytic pocket, a catalytic domain composed of ~160 amino acids, and except for matrilysin and matrilysin-2, a hemopexin-like domain comprised of ~200 amino acids (for review, see Ref. 54). In general, MMP hemopexin domain function is poorly characterized. We are therefore currently elucidating the function of the enamelysin hemopexin domain by inserting an enamelysin transgene that encodes all but the hemopexin domain into the null mouse background. Thus, the enamelysin null mouse may allow us an opportunity to identify functional aspects of specific MMP domains as the tooth develops.

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