The Receptor Activator of Nuclear Factor-κB Ligand-mediated Osteoclastogenic Pathway Is Elevated in Amelogenin-null Mice*

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Ameloblasts synthesize and secrete amelogenins into the dental enamel matrix that undergo systematic proteolysis during enamel mineralization. Numerous mutations were found in the amelogenin coding sequences in patients with the most common genetic disorder affecting enamel, amelogenesis imperfecta (1–5). The targeted disruption of the amelogenin gene locus in mice also showed a hypoplastic enamel phenotype similar to amelogenesis imperfecta, confirming an important role of amelogenins in enamel formation (6).

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In addition to their role in enamel formation, amelogenins are also believed to play a key role in the formation of root cementum, a mineralized layer on the surface of root dentin (7, 8). During cementogenesis, Hertwig’s epithelial root sheath dissociates to form cell aggregates (epithelial rests of Malassez) that are located between the alveolar bone and the tooth root. The mesenchyme-derived cementoblasts secrete cementum matrix onto the root surface to form cementum. The presence of amelogenins was detected on the tooth root surface close to the site of acellular cementum (9) and in the epithelial remnants of the root sheath in rat molars (10), indicating their potential role during cementogenesis.

The present study was undertaken to investigate the expression of two alternate splice forms of amelogenins, M180 and the leucine-rich amelogenin peptide (LRAP), in the periodontal ligament of mouse tooth roots. Lack of M180 and LRAP mRNA expression correlated with cementum defects observed in the amelogenin-null mice. The cementum defects were characterized by an increased presence of multinucleated cells, osteoclasts, and cementicles. These defects were associated with an increased expression of the receptor activator of the nuclear factor-κB ligand (RANKL), a critical regulator of osteoclastogenesis. These findings indicate that the amelogenin splice variants, M180 and LRAP, are critical in preventing abnormal resorption of cementum.

Amelogenin-null Mice—Amelogenin-null mice were generated by gene targeting (6), housed in a pathogen-free animal facility, and fed a dough diet (Bio-Serv, Holton Industries Co., Frenchtown, NJ) and autoclaved water ad libitum. Standard National Institutes of Health guidelines were followed to monitor the health status of the mice and for housing and breeding practices.

Preparation of Tissue Sections—Amelogenin-null mice and wild-type controls 1-day-, 6-month-, and 1-year-old were used for the present study. Eight mice in each group were anesthetized and perfused with 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS), pH 7.4. After dissection, the skulls were fixed in 4% paraformaldehyde for 24 h, decalcified in 10% EDTA and 0.01M PBS (pH 7.4) for 4–6 weeks at 4 °C, dehydrated in a graded series of ethanol, embedded in paraffin, and serially sectioned into coronal sections at 8-μm thickness. The sections were stained with hematoxylin and eosin for histological evaluation.

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Amelogenins Regulate Osteoclastogenesis

TABLE I

| Specificity | Oligonucleotide sequence (5′-3′) | Residues | GenBank accession no. | Product size (bp) | Annealing temperature | Cycle no. |
|-------------|----------------------------------|----------|----------------------|------------------|----------------------|-----------|
| Amelogenin  | Forward AATTGGAAGCCTGTTAGTTTGTTG | 59-81    | D31768                | —                 | 64°C                 | 30        |
|             | Reverse TCAGCTTGCTGGTGTGCTGCCTG  | 614-637  |                      |                  |                      |           |
| Enamelin    | Forward CACTTATATCCACTACAATCCCCTG| 3115-3139| NM017468              | 544               | 58°                  | 26        |
|             | Reverse GGCGTTTTTTTGGCCAGAGAGAG| 3638-3659|                      |                  |                      |           |
| RANKL       | Forward GCTGGCGGAATTCTGGAATTT    | 957-976  | AF053713              | 812               | 58°                  | 30        |
|             | Reverse GGAAATACCTAATGGCAGGAGAGG| 1749-1769|                      |                  |                      |           |
| OPG         | Forward ACCCACACAGAGGAGCCTTT     | 1159-1178| AB1388                | 269               | 60°                  | 26        |
| TRAF 6      | Forward GAGAGATGAGCCGCTACGGA    | 1409-1428|                      |                  |                      |           |
| GAPDHa      | Forward ATGACTTGGATGATCCTGAGA    | 410-429  | NM009424              | 292               | 58°                  | 30        |
|             | Reverse GCATGCATGTGTTGATAGAG    | 702-721  |                      |                  |                      |           |
|             | Reverse GCATGACTGTGGATAGAG      | 298-321  |                      |                  |                      |           |
|             | Reverse GCATGACTGTGGATAGAG      | 562-581  |                      |                  |                      |           |

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| Enamelin    | Forward CACTTATATCCACTACAATCCCCTG| 3115-3139| NM017468              | 544               | 58°                  | 26        |
|             | Reverse GGCGTTTTTTTGGCCAGAGAGAG| 3638-3659|                      |                  |                      |           |
| RANKL       | Forward GCTGGCGGAATTCTGGAATTT | 957-976  | AF053713              | 812               | 58°                  | 30        |
|             | Reverse GGAAATACCTAATGGCAGGAGAGG| 1749-1769|                      |                  |                      |           |
| OPG         | Forward ACCCACACAGAGGAGCCTTT | 1159-1178| AB1388                | 269               | 60°                  | 26        |
| TRAF 6      | Forward GAGAGATGAGCCGCTACGGA | 1409-1428|                      |                  |                      |           |
| GAPDHb      | Forward ATGACTTGGATGATCCTGAGA | 410-429  | NM009424              | 292               | 58°                  | 30        |
|             | Reverse GCATGACTGTGGATAGAG    | 702-721  |                      |                  |                      |           |
|             | Reverse GCATGACTGTGGATAGAG    | 298-321  |                      |                  |                      |           |
|             | Reverse GCATGACTGTGGATAGAG    | 562-581  |                      |                  |                      |           |

a Product sizes of major bands are 601, 576, 496, 256, and 221 bp.
b GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

were stained with hematoxylin and eosin using standard protocols. Frozen sections from EDTA-decalcified skulls of three mice in each group were stained for tartrate-resistant acid phosphatase (TRAP) activity with the leukocyte acid phosphatase kit (Sigma).

Immunohistochemistry—Frozen sections from the wild-type and amelogenin-null skulls were immunostained for RANKL using goat polyclonal antibodies against mouse RANKL (R&D Systems, Minneapolis, MN) overnight at 4°C. After washing in PBS, the sections were incubated with peroxidase-conjugated mouse antibodies against goat IgG (Vector Laboratories, Burlingame, CA). To visualize the immunoreactant, the sections were treated with diaminobenzidine substrate and counterstained with hematoxylin for light microscopy.

Quantitative Micrograph Analysis—Light micrographs of the sections stained with hematoxylin and eosin were used for the histomorphometric measurements. Twenty slides, each with three sections of the first two mandibular molars from each mouse, were used for counting defects in cementum and dentin (e.g. multinuclear cells and cementicle numbers). Each number on the histogram represents the mean ± S.D. of observations from either the first or second mandibular molar from a total of four mice.

Scanning Electron Microscopy Analysis of Molar Teeth—Molars from the wild-type and amelogenin-null mice were photographed using scanning electron microscopy at 20 kV (Jeol JSM T330A, Jeol, Inc., Peabody, MA).

RNA Isolation and Gene Expression Analysis by RT-PCR—CM/PDL cell populations were established from cells lining the tooth root surface of 6-month-old wild-type and amelogenin-null mice. Mandibular first molars were extracted by dissecting the molars with adherent PDL from surrounding alveolar bone. The CM/PDL cells were isolated from the surface of the mandibular molars as described (18, 19). Briefly, molars were placed in a 1.5 ml centrifuge tube containing PBS with 1 mg/ml collagenase ( Worthington, Lakewood, NJ) and 0.25% trypsin-EDTA (Invitrogen) and incubated for 2 h at 37°C. Mandibular first molar tooth germs from 1-day-old wild-type and amelogenin-null mice were dissected from surrounding tissues. Total RNA was isolated using the RNA isolation kit (Stratagene) and treated with DNase. The RNA samples (1 μg each) were subjected to first strand cDNA synthesis using the SuperScript™ reverse transcriptase from Invitrogen. RT-PCR was performed using gene-specific primers as described in Table I. All PCR reactions were carried out in a PerkinElmer gene PCR system 600. PCR products were cloned into a T-vector (Promega, Madison, WI), and the nucleotide sequences were determined. The two amplified products (576 and 221 bp) that were detected in the CM/PDL cells contained exons 2, 3, 5, and 6 and exons 2, 3, 5, and partial 6, respectively (Fig 1B).
sequences were in agreement with the reported amelogenin splice forms (20–22). The amelogenins derived from these spliced forms were identified previously as M180 and M59 (LRAP) in the ameloblasts. Enamelin, one of the enamel matrix proteins expressed in ameloblasts, was expressed in wild-type and the null tooth buds but not in the CM/PDL cell populations.

Increased Cementum and Dentin Defects in Amelogenin-null Mice—The wild-type mice did not show any significant difference in the cementum thickness or abnormalities in the pulp and surrounding bone regions (Fig. 2A). However, the cementum of the null mice displayed resorptive lacunae at sites where periodontal ligaments attach to the cementum surface (Fig. 2B and D). Multiple intrusive attachments of PDL extended through the cementum into the root dentin of the amelogenin-null mice. Furthermore, we examined the surface of the root cementum of the amelogenin-null mice by scanning electron microscopy. The molar root surface of the wild-type mice showed a relatively smooth surface with shallow cavities caused by Sharpey’s fibers. F, scanning electron microscopy analysis of the tooth root of an amelogenin-null mouse showed resorptive lacunae (arrows) on the root surface. Scanning electron microscopy analysis of fractured teeth from wild-type (G) and amelogenin-null (H) mice shows the depth of resorptive lacunae (arrows). b, bone; c, cementum; d, dentin; pdl, periodontal ligament; pu, pulp. Scale bars for panels A–D, 100 μm; panels E–H, 10 μm.

Fig. 2. Cementum defects in the amelogenin-null mice. Sagittal sections of the mandibular second molar of wild-type (WT) (A) and amelogenin-null mice (KO) (B) stained with hematoxylin and eosin (note cuspal attrition as indicated by arrowheads). C and D, higher magnification of the indicated root area (boxes) in panels A and B (note resorptive lacunae penetrating into cementum and dentin as indicated by arrows). E, scanning electron microscopy analysis of the tooth roots of a wild-type mouse showing a relatively smooth surface with shallow cavities caused by Sharpey’s fibers. F, scanning electron microscopy analysis of the tooth root of an amelogenin-null mouse showing resorptive lacunae (arrows) on the root surface. Scanning electron microscopy analysis of fractured teeth from wild-type (G) and amelogenin-null (H) mice shows the depth of resorptive lacunae (arrows). b, bone; c, cementum; d, dentin; pdl, periodontal ligament; pu, pulp. Scale bars for panels A–D, 100 μm; panels E–H, 10 μm.

We further quantitated the cementum and dentin defects in the null mice (Fig. 3B) and compared them with the wild-type mice (Fig. 3A). As described under “Experimental Procedures,” 20 sagittal (serial) sections from three wild-type and three amelogenin-null mice, each at 6 months and 1 year of age, were stained with hematoxylin and eosin and counted for all resorptive lacunae. The first and second molars both showed a pattern of progressive increase in cementum defects (Fig. 3C). The amelogenin-null mice displayed four times more cementum defects at both 6 months and 1 year of age as compared with wild-type mice. Similarly, in the first and second molars, 13 times more root dentin defects were observed at both 6 months and 1 year of age as compared with wild-type mice (Fig. 3D).

Increased Presence of Multinucleated Cells in Amelogenin-null Mouse Teeth—As compared with the wild-type mouse tooth roots (Fig. 4C), many multinucleated cells were observed in the cementum and dentin regions of the null tooth roots (Fig.
FIG. 4. Increased number of osteoclasts/odontoclasts in amelogenin-null mice. Tartrate-resistant alkaline phosphatase staining of sagittal tooth sections from 6-month-old wild-type (WT) (A) and amelogenin-null (KO) (B) mice (note that positive cells, as marked by arrows, appear in close proximity to cementum, indicating elevated osteoclastogenesis activity). Hematoxylin and eosin stained sagittal tooth sections from 6-month-old wild-type (C) and amelogenin-null (D) mice. Wild-type mice show normal PDL cells in tooth roots, whereas the amelogenin-null PDL cells showed more intense multinucleated cells. E, multinucleated cells in the periodontal region of the first (Molar 1) and second (Molar 2) molars from 6-month- and 1-year-old wild-type and amelogenin-null mice were counted and presented as a histogram. Values represent mean ± S.D. of observations from three mice. Asterisks denote statistically significant differences (*, p < 0.05; **, p < 0.01). c, cementum; d, dentin; pdl, periodontal ligament; pu, pulp. Scale bar (panels A–D), 100 μm.

4D). The amelogenin-null mice displayed a 2-fold increase in number of the multinucleated cells as compared with wild-type mice at both 6 months and 1 year of age (Fig. 4E). Although the null mice had significantly more multinucleated cells than the wild-type mice, they did not show any progressive increase in number with age. Interestingly, these cells were stained positive for TRAP activity, indicative of their osteoclastic/odontoclastic nature (Fig. 4B), whereas wild-type mice did not display similar TRAP activity (Fig. 4A).

Increased Osteoclastogenesis Near the Roots of Amelogenin-null Mouse Teeth—To correlate tooth root defects with the osteoclastogenic deregulation in CM/PDL cells of amelogenin-null mice, we examined the expression of RANKL, osteoprotegerin (OPG), and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF 6) by RT-PCR and immunohistochemical analysis. RT-PCR analysis revealed that RANKL and TRAF 6 mRNA levels were significantly elevated in CM/PDL cells of the

null mice (Fig. 5A). However, the expression of OPG, an orphan receptor for RANKL, was not altered. The distribution of RANKL in the periodontal tissue of amelogenin-null mice was also examined by immunohistochemical analysis. RANKL immunoreactivity was not detected in the PDL cells of wild-type teeth (Fig. 5B). In contrast, the amelogenin-null PDL cells showed more intense staining for RANKL near the bone and cementum surface (Fig. 5C). These observations suggest that the abnormal localization of osteoclasts close to the tooth root correlates with the accelerated resorption of cementum in the amelogenin-null mice.

Increased Cementicles at the Periodontal Ligament Space in Amelogenin-null Mice—The progressive occurrence of cementicles adhering to the cementum surface in the amelogenin-null mice indicates a defect in periodontal tissue and possibly a "hypercementosis-like" condition (Fig. 6B). The amelogenin-null mice displayed 2–4 times more cementicles near the molar tooth root at 6 and 12 months of age (Fig. 6C). The presence of cementicles is well documented in the periodontal spaces in pathological conditions as well as in aging humans. The increased presence of these cementicles confirms abnormal cementum in amelogenin-null mice.

DISCUSSION

Amelogenins, highly conserved proteins that constitute 90% of the enamel organic matrix, are produced by ameloblasts shortly before tooth eruption. Numerous experimental approaches have indicated that amelogenins play an important role in amelogenesis (6, 23, 24). Although specific amelogenin splice products have been implicated in tissue-specific epithelial-mesenchymal signaling during tooth development (20–22, 25), the distribution of specific splice forms and the precise functions associated with the individual peptides are still unclear. The implied but undefined role of amelogenins in cemen-
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Fig. 6. Increased number of cementicles in amelogenin-null mice. Sagittal section of the mandibular second molar of wild-type (WT) (A) and amelogenin-null (KO) (B) mice stained with hematoxylin and eosin (note cementicles as indicated by arrows). C, total number of cementicles in the first (Molar 1) and second (Molar 2) molars from 6-month- and 1-year-old wild-type and amelogenin-null mice were counted and presented as a histogram. Values represent mean ± S.D. of observations from three mice. Asterisks denote statistically significant differences (⁎⁎, p < 0.01). a, bone; b, cementum; c, dentin; pdl, periodontal ligament. Scale bar (panels A and B), 100 µm.

Amelogenins form the basis of the present study. During tooth development, at least nine different mRNA splice forms are generated from the amelogenin gene as a result of alternate splicing (26). Interestingly, the presence of amelogenins in the tooth root region has been detected only by immunohistochemical and *in situ* hybridization studies (9, 10, 27). However, these studies could not identify the presence of individual alternate splice forms of mRNA or their translational products. Unlike unerupted molar teeth, the CM/PDL cells from the adult wild-type and amelogenin-null mice were counted and presented as a histogram. Values represent mean ± S.D. of observations from three mice. Asterisks denote statistically significant differences (⁎⁎, p < 0.01). a, bone; b, cementum; c, dentin; pdl, periodontal ligament. Scale bar (panels A and B), 100 µm.

A detailed analysis of the amelogenin-null mice revealed normal cementogenesis but poor maintenance of the cementum, as observed by the increased presence of tooth root resorption. Dentin, cementum, and enamel of permanent teeth undergo resorption. Dentin, cementum, and enamel of permanent teeth show increased resorption of the cementum. Amelogenin-null mice exhibit more resorption of the cementum than surrounding alveolar bone. The presence of multinucleated cells and TRAP-positive cells near the cementum and close to the lacunae, indicate their potential involvement in the cementum and dentin resorption process through the osteoclastogenic pathway.

RANKL-mediated signaling is one of the mechanisms by which osteoclastogenesis is regulated. Bone resorption by active osteoclastogenesis requires the expression of RANKL, RANK, OPG, and TRAF 6 (32–35). RANKL is produced by osteoblasts and bone marrow stromal cells (36, 37) and interacts with its receptor RANK during active osteoclastogenesis. In contrast, OPG, a soluble decoy receptor, competes with RANK for RANKL binding (38–40) and serves as an inhibitor of osteoclastogenesis. TRAF 6 is downstream in the RANKL/RANK pathway [41, 42]. The PDL cells express both RANKL and OPG (32–35, 41, 43) and enhance the resorptive activity of the osteoclasts that differentiate from peripheral blood mononuclear cells (PBMCs) through cell-to-cell contact. However, OPG prevents the cell-to-cell contact by binding to RANKL. Consistent with the increased resorption of the cementum, RANKL expression was significantly elevated and increasingly immunolocalized near the cementum of the amelogenin-null mice. However, OPG expression remained unaltered. The increased TRAF 6 and number of osteoclasts in the amelogenin-null mice suggest enhanced RANKL-mediated differentiation, resulting in active resorptive processes.

In addition to the cementum resorption, the tooth roots of amelogenin-null mice exhibited increased numbers of cementicles adhering to the surface of the cementum. Recent reports showed calcified bodies known as psammoma-like ossicles and cementicles in the osteoblastoma and juvenile ossifying fibroma of the craniofacial skeleton (44, 45). In rare pathological conditions, a large number of cementicles may fuse together to give rise to an odontogenic tumor (45, 46). These cementicles were also observed in human aging. Aging is likely to augment orthodontic movements, resulting in trauma to the tooth roots as seen in the aging senescence-accelerated mice (47). Despite the increased presence of cementicles in pathological conditions and aging, their precise involvement in such conditions, as a cause or a consequence, is not well understood.

It is well established that amelogenins are predominantly expressed in the ameloblasts and regulate the biomineralization of enamel. The expression of amelogenins has also been reported in the odontoblasts in molar tooth roots; however, their precise functions were not established (9, 10, 48, 49). Recent reports have indicated that the amelogenins, mainly M180 and LRAP, induce bone formation in vitro (25, 26). Similarly, the expression of amelogenins in odontoblasts was also implicated in reciprocal signaling between pre-ameloblasts and pre-odontoblasts during tooth development. It appears from these experiments that amelogenins may be essential in regulating the critical balance between osteoblastic and osteoclastic activity in bone remodeling. The expression of only M180 and LRAP in the periodontal region further supports the hypothesis that, in addition to enamel mineralization, amelogenins may have other functions. Increased RANKL pathway expression in the absence of amelogenins in the periodontal region indicates that amelogenins play a key role in the regulation of the osteoclastogenic pathway. Unlike clinical orthodontic movements, the resorptive phenomenon observed in the amelogenin-null tooth roots is more preferential toward cementum than alveolar bone. The restricted expression of amelogenins in the periodontal region between the alveolar bone and the cementum by the epithelial rests of Malassez indicate that amelogenins may prevent abnormal resorption of cementum.

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