Published in final edited form as:

J Dent Res. 2008 July ; 87(7): 624–629.

Periodontal Breakdown in the *Dmp1* Null Mouse Model of Hypophosphatemic Rickets

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Abstract

Dentin Matrix Protein 1 (DMP1) is highly expressed in alveolar bone and cementum, which are important components of the periodontium. Therefore, we hypothesized that *Dmp1* is critical for the integrity of the periodontium, and that deletion may lead to increased susceptibility to disease. An early-onset periodontal defect was observed in the *Dmp1* null mouse, a mouse model of hypophosphatemic rickets. The alveolar bone is porous, with increased proteoglycan expression. The cementum is also defective, as characterized by irregular, punctate fluorochrome labeling and elevated proteoglycan. The osteocyte and cementocyte lacuno-canalicular system of both alveolar bone and cementum is abnormal, with irregular lacunar walls and fewer canaliculi. As a consequence, there is significant interproximal alveolar bone loss, combined with detachment between the periodontal ligament (PDL) and cementum. We propose that defective alveolar bone and cementum may account for the periodontal breakdown and increased susceptibility to bacterial infection in *Dmp1* null mice.

Keywords

DMP1; PDL; cementum; hypophosphatemic rickets; alveolar bone

INTRODUCTION

Hypophosphatemic rickets is characterized by osteomalacia, resulting in limb deformities due to phosphate wasting. Recently, we have shown that mutation or deletion of *Dmp1* leads to hypophosphatemic rickets (Feng *et al.*, 2006). Persons with hypophosphatemic rickets have enlarged pulp chambers and increased predentin width (Goodman *et al.*, 1998; Murayama *et al.*, 2000; Pereira *et al.*, 2004), consistent with what we have described previously in the *Dmp1* null mouse (Ye *et al.*, 2004). Immature calcospherites have been described in the dentin of hypophosphatemic rickets, suggesting delayed mineralization (Boukpepsi *et al.*, 2006).

Dentin Matrix Protein 1 (DMP1), an acidic phosphorylated extracellular matrix protein, was first isolated from dentin (George *et al.*, 1993), and later from bone, cartilage, and cementum (MacDougall *et al.*, 1998; Butler *et al.*, 2002; Feng *et al.*, 2002; Toyosawa *et al.*, 2004). Although DMP1 is expressed in all mineralized tissues, the highest expression appears in

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A supplemental appendix to this article is published electronically only at <http://jdr.iadrjournals.org/cgi/content/full/87/7/624/DC1>.

osteocytes (Toyosawa *et al.*, 2004). Recently, broader but much lower DMP1 expression has been reported in non-mineralized tissue, such as brain, kidney, and pancreas (Terasawa *et al.*, 2004). DMP1 may function to induce differentiation of mesenchymal cells to odontoblast-like cells, thereby enhancing mineralization (Narayanan *et al.*, 2001), and by functioning as a transcription factor to target the nucleus and activate osteoblast-specific genes (Narayanan *et al.*, 2003).

We originally generated *Dmp1* null mice to study the function of this gene (Feng *et al.*, 2003, 2006; Ye *et al.*, 2004, 2005). The null mice are normal at birth, but post-natally develop a profound tooth phenotype, such as enlarged pulp chambers and increased width of the predentin zone with reduced dentin thickness (Ye *et al.*, 2004). Since DMP1 is highly expressed in cementum and alveolar bone, we sought to determine if deletion of this protein in these tissues is responsible for any periodontal defects. Our hypothesis was that DMP1 is critical for the integrity of alveolar bone and cementum, and that deletion of DMP1 in these tissues would lead to increased susceptibility to disease.

MATERIALS & METHODS

Animals

Mice deficient in *Dmp1* were generated as described previously (Feng *et al.*, 2003). To obtain *Dmp1* null mice, we interbred *Dmp1* heterozygous mice to generate homozygotes in the C57BL/6 background. In this paper, wild-type mice are also designated as WT; *Dmp1* null mice are designated as KO. The animal protocol was approved by Institutional Animal Care and Use Committees at the University of Missouri-Kansas City.

Radiography and Micro-computed Tomography (Micro-CT)

Mandibles were examined on a Faxitron MX-20 Radiography System (Faxitron X-ray Corp., Buffalo Grove, IL, USA). Three-dimensional images of 12-month mandibles from WT and *Dmp1* null mice were scanned with Micro-CT. Images were reconstructed with EVS Beam software with a global threshold at 1400 Hounsfield Units.

Resin Infiltrating and Acid-etching

To study the canalicular system of osteocytes, we polished the surface of methylmethacrylate-infiltrated alveolar bone. The surface was then acid-etched with 37% phosphoric acid for 2–10 sec and 5% sodium hypochlorite for 5 min, then coated with gold and palladium, and examined by means of a FEI/Philips XL30 Field Emission Environmental Scanning Electron Microscope (SEM) (Hillsboro, OR, USA) (Martin *et al.*, 1978; Feng *et al.*, 2006).

Histology and Histomorphometric Analysis

Mandibles from WT and *Dmp1* null mice were fixed in 4% paraformaldehyde, followed by decalcification in 10% EDTA solution for 3 wks. Sections were stained with hematoxylin & eosin (H&E). Histomorphometric analysis was carried out with the Image Analysis System (AnalySIS, Lakewood, CO, USA). Three sections, at least 24 μ m apart, were examined from each animal (5 animals in each group). Alveolar bone area and bone width were measured as reported (Barnett and Rowe, 1986; Nociti *et al.*, 2001). Specifically, the alveolar bone area is defined as the interproximal bone area above the straight line connecting the root apices of the first and second mandibular molars. The alveolar bone width is measured at the coronal, middle, and apical thirds of interproximal bone between the first and second molars, respectively, and the mean value is used as the actual bone width. All results were expressed as mean $\pm$ standard deviation (SD).

Fluorochrome Labeling of Cementum

To analyze cementum formation rate, we injected mice 5 days apart with calcein (5 mg/kg, Sigma-Aldrich, St. Louis, MO, USA; intraperitoneal injection, i.p.), alizarin red (20 mg/kg, Sigma-Aldrich, i.p.), and again with calcein. The animals were killed 2 days after the final injection.

Immunohistochemistry

Immunostaining was performed as described previously (Ye *et al.*, 2004), with rabbit polyclonal antibodies for DMP1 (Larmas *et al.*, 2008), biglycan and decorin (gifts from Dr. Larry Fisher, NIDCR, Bethesda, MD, USA). We obtained negative controls by replacing the primary antibody with rabbit serum (Appendix Fig. 1).

Statistical Analysis

Data from the histomorphometric analysis were analyzed by Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

Dmp1 Null Mice Develop Early-onset Periodontal Breakdown

Unlike humans, mice do not naturally develop periodontal disease until extreme old age, usually older than 1 yr, and mainly in the maxilla (Page and Schroeder, 1982). In contrast, *Dmp1* null mice develop an early-onset periodontal defect (Fig. 1A). At the age of 3 wks, the *Dmp1* null alveolar bone appears grossly normal in shape, although the bone is woven and immature in nature. However, at ages 3 and 6 mos, there is a severe bone defect, combined with periodontal ligament (PDL)/cementum detachment in *Dmp1* null mice (Fig. 1A, lower panels). Quantitative histomorphometric analysis shows significant decrease of bone in KO mice at 3 mos, but not at 3 wks (Fig. 1B). There is no difference in bone height between WT and *Dmp1* null mice (data not shown), suggesting that the bone defect in *Dmp1* null interproximal bone is mainly vertical.

Dmp1 Null Mice Display Porous, Hypomineralized, Immature Alveolar Bone

Although signs of dentin defects, as reflected by enlarged pulp and thin dentin, were obvious at the age of 3 wks, the bone between the first and second molars appeared comparable between the WT and *Dmp1* null mice (Fig. 2A, left lower panel, black arrows). By the age of 3 mos, the *Dmp1* null alveolar bone appeared porous compared with that of the age-matched control (Fig. 2A, middle lower panel, black arrow). At the age of 6 mos, the bone defect was more striking (Fig. 2A, right lower panel, white arrow), and by the age of 12 mos, the bone became extremely porous, as determined by Micro-CT. Also note the misalignment of teeth due to severe bone loss. Histological sections from 3-week and 3-month *Dmp1* null alveolar bone reveal immature woven bone, with more osteoblasts and bone marrow spaces, suggesting that DMP1 is required for maturation of woven bone into lamellar bone (Appendix Fig. 2).

Increased Proteoglycans and Irregular Osteocyte Lacuno-canalicular System in Alveolar Bone

Biglycan and decorin are members of a small leucine-rich proteoglycan family. Elevated biglycan and decorin lead to mineralization defects in the dentin sialophosphoprotein null tooth (Sreenath *et al.*, 2003), and elevated biglycan protein expression is also observed in the *Dmp1* null predentin (Ye *et al.*, 2004). To address whether there are any changes in biglycan or decorin expression in the *Dmp1* null alveolar bone, we used polyclonal antibodies to mouse biglycan or decorin (kind gifts from Dr. Larry Fisher, NIDCR) for immunostaining of the interproximal bone in 3-week- and 3-month-old mice. Dramatic increases in biglycan and

decorin immunostaining were observed in the *Dmp1* null alveolar bone (Fig. 3A, lower panels, white asterisks), suggesting either increased accumulation of these extracellular matrix proteins or greater exposure due to hypomineralization.

Previously, we had shown a retardation of maturation of *Dmp1* null osteoblasts into osteocytes (Feng *et al.*, 2006). For further study of the morphological changes of the osteocyte lacuno-canalicular system in the *Dmp1* null alveolar bone, resin-infiltrated sections were acid-etched to reveal a three-dimensional relief of the osteocyte lacuno-canalicular system, by two-dimensional SEM. Images showed that the WT osteocyte lacunae were highly organized and spaced regularly, generally in linear arrays. The canaliculi were generally straight and ran perpendicular to the long axis of the osteocyte, with numerous canaliculi with smooth walls (Fig. 3B, left panels). In contrast, the *Dmp1* null osteocyte lacunae appeared to have rough walls, and were larger in size, with fewer canaliculi. The distribution of the lacunae appeared less organized and more clustered, while their canaliculi were less straight and smooth (Fig. 3B, right panels). The abnormal morphology of lacunae in the *Dmp1* null mice suggests that DMP1 plays a role in the formation of the lacuno-canalicular system.

Abnormal Cementum and PDL in *Dmp1* Null Mice

When the cementum and PDL were compared between *Dmp1* null and WT mice by H&E staining, it was noted that the PDL cells were irregularly shaped. In the WT mice, the cuboid-shaped cementoblasts (Fig. 4A, left panel, red arrows) were regularly aligned on the surface of cementum, and a clear acellular cementum layer was present (Fig. 4A, left panel). In contrast, the acellular cementum layer in *Dmp1* null mice was much thinner and more difficult to recognize. Second, there were few *Dmp1* null cementoblasts, and they were no longer cuboid, but spindle-shaped (Fig. 4A, right panel). Third, the WT PDL layer was composed of different cell types, whereas the KO PDL layer was composed of fewer and spindle-shaped fibroblasts.

Previously, using *Dmp1*-lacZ knock-in mice, we reported that DMP1 is expressed in cementum (Feng *et al.*, 2003). Using immunostaining for DMP1, we showed that the protein was also expressed in both acellular (red arrowhead) and cellular cementum (red arrow), as well as in alveolar bone (Fig. 4B, brown). Similar to alveolar bone, increases in biglycan and decorin were observed in the cellular cementum (Fig. 4C, lower panels, white asterisks). These proteoglycans may potentially cause or be the result of the defective process.

Next, the cementocyte lacunar morphology was compared between the WT and KO, by resin-infiltrating and acid-etching techniques. Similar to the abnormal KO osteocyte lacunae, the *Dmp1* null cementocyte lacuna is rough on the lacunar wall, with few dendrites (Appendix Fig. 3). To evaluate the change of cementum formation rate, we performed fluorochrome injections and showed distinct labeling lines in WT cementum. In contrast, the labeling lines in *Dmp1* null mice were sparse and diffuse (Appendix Fig. 3, white arrow), suggesting defective calcospherite maturation in the KO mice.

DISCUSSION

We have reported that *Dmp1* null mice post-natally develop a profound tooth phenotype characterized by a partial failure of maturation of predentin into dentin, enlarged pulp chambers, and increased width of the predentin zone with a reduced dentin wall (Ye *et al.*, 2004). In this study, we show that *Dmp1* null mice develop severe alveolar bone and cementum defects, which may explain the early periodontal breakdown in these mice. DMP1 is highly expressed in both alveolar bone and cementum. Loss of *Dmp1* results in alveolar bone that is porous and immature, with a large amount of osteoid similar to that shown in bone of the appendicular skeleton (Feng *et al.*, 2006). Cementum, a similar bony tissue, shows abnormal

mineralization, with immature calcospherites as shown in bone (Feng *et al.*, 2006) and in the dentin of hypophosphatemic individuals (Boukpepsi *et al.*, 2006).

Elevated protein expression of both biglycan and decorin was observed in both alveolar bone and cementum in the *Dmp1* null mice. Biglycan and decorin are small leucine-rich proteoglycans, produced by osteoblasts and odontoblasts, and lack of these proteoglycans has been associated with defective mineralization in dentin (Sreenath *et al.*, 2003). Decorin is also abundant in unmineralized bone matrices in the embryonic rat mandible, but could not be detected in mineralized matrices, suggesting that decreased proteoglycan is associated with normal matrix mineralization (Kamiya *et al.*, 2001). *In vitro* studies have shown that recombinant decorin and biglycan can inhibit hydroxyapatite-induced crystal growth (Boskey *et al.*, 1997; Sugars *et al.*, 2003). Elevated expression of these proteoglycans in the matrix from *Dmp1* null mice may be partially responsible for defective mineralization in alveolar bone and cementum.

Although bacteria play an important role in the development of periodontitis in the clinical patient, this does not appear to be the case in the *Dmp1* null mice. Significant vertical bone defect is evident in the *Dmp1* null mice as early as 3 mos, without any clear evidence of bacterial infection or inflammatory response. With increasing age (up to 12 mos; Appendix Fig. 4), *Dmp1* null mice are more prone to bacterial infection, most likely due to alveolar bone defect and detachment between the PDL and cementum. Therefore, bacteria may not play a major role in the formation of the periodontal defects in *Dmp1* null mice. However, to confirm this hypothesis, future experiments using antibiotics or a bacteria-free environment are planned.

It is of note that PDL cells in the *Dmp1* null mouse have an abnormal spindle shape. We do not know if this is caused by an intrinsic defect in the PDL cells and/or is secondary to the defects in bone and cementum. However, immunostaining of tissue from the null mice did not reveal detectable expression of DMP1 in PDL cells, whereas significant DMP1 expression was observed in alveolar bone and cementum. Second, the *Dmp1* null mice showed comparable alveolar bone and cementum at 3 wks, suggesting that PDLs in the *Dmp1* null mouse still have osteogenic or cementogenic capability; therefore, we propose that their abnormal spindle shape may be secondary to the bone and cementum defects. Further *in vitro* primary PDL cell culture experimentation is needed to answer this question.

DMP1 expression increases selectively after mechanical loading in the tooth movement model (Gluhak-Heinrich *et al.*, 2003) and in the ulna of loaded mice (Yang *et al.*, 2005). Loading of bone in the *Dmp1* null mouse produces more strain than in wild-type or heterozygous littermates under identical mechanical loads (Rios *et al.*, 2005). These observations support the concept that DMP1 may be an important factor in the regulation of the osteocyte response to load. Both osteocyte and cementocyte lacunae were abnormal in these mice, and the canaliculi were reduced in number, suggesting abnormal bone fluid flow through these systems. Therefore, ablation of DMP1 may lead to pathological response to physiological mechanical loading, such as mastication. Overloading of the alveolar bone may also generate excessive microdamage, leading to greater bone loss (Frost, 1992, 1994). This, combined with the lack of support by the defective alveolar bone and cementum, may exacerbate bony defects.

In summary, we report severe alveolar bone and cementum defects in *Dmp1* null mice most likely responsible for periodontal breakdown, especially in the interproximal area between molars, which worsens with age. Although these defects are distinct from clinical periodontitis, analysis of our data suggests that, without proper phosphate treatment and dental care, hypophosphatemic individuals may be predisposed to bacterial infection.

Acknowledgements

This research is supported by NIDCR grants DE016977 and DE07294, and by NIAMS grants AR51587 and AR46798.

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Figure 1.

Dmp1 null mice develop early periodontal breakdown. **(A)** Histological pictures of interproximal areas between the first and second lower molars at different stages (3 wks, 3 and 6 mos). At 3 wks of age, the periodontal attachment is intact in the *Dmp1* null mice. At 3 mos, the alveolar bone defect becomes evident, accompanied by detachment between the PDL and cementum, compared with the healthy periodontal tissue in WT control mice. At 6 mos, the periodontal defect becomes more severe in the KO mice. **(B)** Quantitative analyses of alveolar bone width and alveolar bone area between the first and second molars from ages of 3 wks to 3 mos, showing decreases with age. +/+, WT; -/-, KO; n = 5; *P < 0.05; **P < 0.01, data represent mean ± SD. **(AQ)**

Figure 2.

Dmp1 null mice have defective alveolar bone. (A) Representative radiographs of mandibles from three-week-, three-month-, and six-month-old mice (WT in upper panels, and KO in lower panels). The porous alveolar bone in the null animals is evident by the ages of 3 and 6 mos (arrows). (B) Representative micro-CT reconstructions of mandibles from 12-month-old animals. Note that there is severe bone loss in KO mice.

Figure 3.

Elevated proteoglycan expression and defective lacuno-canalicular morphology in *Dmp1* null alveolar bone. (A) Immunostaining for biglycan and decorin on sections of alveolar bones from three-week- and three-month-old animals (WT in the upper panels, and KO in the lower panels). The increased staining of biglycan and decorin is evident (white asterisks). (B) SEM images of resin-infiltrated, acid-etched alveolar bone from three-week-old WT (left panels) and KO mice (right panels). Osteocyte lacunae were filled with resin, and the mineral was removed by 37% phosphoric acid and 5% sodium hypochlorite. The smooth canalicular walls from a representative lacuna are indicated with red arrows (lower left panel), while rough canalicular walls from a lacuna in KO bone are indicated by arrowheads (lower right panel). Note that the

WT osteocyte lacuno-canalicular system is evenly distributed, but the KO osteocyte lacuno-canalicular system is disorganized, and lacunae are clustered. There is also a large amount of osteoid present in the matrix.

Figure 4.

Cementum and PDL in the *Dmp1* null mouse are malformed. (A) H&E staining of section containing cementum, cementoblasts, PDL. The cuboidal cementoblasts aligned along the surface of the cementum in the WT (left panel), as indicated by the red arrows, are absent in the KO (right panel). Note that the cells in the WT PDL are mixed, with few spindle-like shapes (left panel), whereas the majority of the KO PDL cells are spindle-shaped (right panel). (B) Immunostaining of a one-month-old mandible for DMP1. Brown signal for DMP1 immunostaining in the acellular layer is indicated by a red arrowhead, and in the cellular layer by a red arrow. (C) Immunostaining with polyclonal antibodies against biglycan and decorin in cellular cementum. The increased expression of biglycan and decorin in KO cellular cementum is indicated by white asterisks in both ages of 3 wks and 3 mos.

Appendix Figure 1.
Negative control images for immunostaining of alveolar bone and cementum.

Appendix Figure 2.

Images of histological sections stained with H&E from three-week- and three-month-old WT (upper panels) and KO (lower panels). WT bone is smooth with few marrow spaces, while the KO bone is immature with large bone marrow spaces and increased numbers of osteoblasts compared with the age-matched WT bone.

Appendix Figure 3.

Dmp, null cementum is malformed. (A) SEM images of resin-infiltrated, acid-etched cementum. The malformed KO cementocyte lacunae with a lack of canaliculi are shown in the lower panel. (B) Fluorochrome labeling of cementum. Three fluorochrome injections were given 5 days apart. The continuous, distinct labeling lines in WT cementum are indicated with a red arrow (upper panel), while the poorly labeled, punctate labeling of calcospherites in the KO cementum is indicated by a white arrow (lower panel).

Appendix Figure 4.

Dmp1 null mice develop bacterial infection only with aging (1 yr), as shown in the H&E-stained tissues from 12-month-old mandibles (WT in the upper left panel and the KO in the other panels). A loss of alveolar bone is indicated by the black arrowhead. The resorption of the cementum and dentin is indicated by black arrows. Bacterial colonies are evident, accompanied by infiltrated lymphocytes and neutrophils. Detritus such as hair shafts is also present, as indicated by white arrows.