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Aberrant Cementum Phenotype Associated With the Hypophosphatemic *Hyp* Mouse

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Abstract

Background—Cementogenesis is sensitive to altered local phosphate levels; thus, we hypothesized a cementum phenotype, likely of decreased formation, would be present in the teeth of X-linked hypophosphatemic (*Hyp*) mice. Mutations in the phosphate-regulating gene with homologies to endopeptidases on the X chromosome (*Phex*) cause X-linked hypophosphatemia, characterized by rickets, osteomalacia, and hypomineralized dentin formation, a phenotype recapitulated in the *Hyp* mouse homolog. Here, we report a developmental study of tooth root formation in *Hyp* mouse molars, focusing on dentin and cementum.

Methods—Light and transmission electron microscopy were used to study molar tissues from wild-type (WT) and *Hyp* mice. Demineralized and hematoxylin and eosin–stained tissues at developmental stages 23 to 96 days postcoital (dpc) were examined by light microscopy. Immunohistochemistry methods were used to detect bone sialoprotein (BSP) distribution in *Hyp* and WT mouse molar tissues, and transmission electron microscopy was used to study similar molar tissues in the non-demineralized state.

Results—Dentin in *Hyp* mice exhibited mineralization defects by 33 dpc, as expected, but this defect was partially corrected by 96 dpc. In support of our hypothesis, a cementum phenotype was detected using a combination of immunohistochemistry and transmission electron microscopy, which included thinner BSP-positive staining within the cementum, discontinuous mineralization, and a globular appearance compared to WT controls.

Conclusion—Mutations in the phosphate-regulating *Phex* gene of the *Hyp* mouse resulted in defective cementum development.

Keywords

Cementum; dentin; metabolism; *Phex*; phosphate; X-linked hypophosphatemic

X-linked hypophosphatemia (XLH), or vitamin D-resistant rickets, is a disorder from inactivating mutations in *Phex* and is the most common non-nutritional form of rickets in developed countries.¹ PHEX, an endopeptidase, is expressed in osteoblasts, osteocytes, and odontoblasts, suggesting a role in biomineralization.² Similar to patients with XLH, the X-

linked hypophosphatemic (*Hyp*) mouse homolog features shortened bones with rickets and osteomalacia.³ Transgenic expression of *Phex* in the *Hyp* mouse restored the bone phenotype without affecting phosphate (P_i) metabolism deficiencies.⁴ P_i wasting in XLH and *Hyp* mice is associated with increased expression of fibroblast growth factor-23 (*Fgf-23*), a phosphatonin that increases renal P_i excretion.⁵ *Hyp* mice are also characterized by reduced 1, 25-(OH)₂D₃ (Vit D₃) levels, increased parathyroid hormone levels, and normal calcium levels.⁴

In addition to skeletal defects, tooth mineralization in XLH and *Hyp* mice is adversely affected, with thin dentin, widened predentin, and “interglobular” dentin indicating hypomineralization.^{6–8} Autosomal recessive hypophosphatemic rickets is another phosphate-wasting disorder featuring tooth defects; it results from mutations in dentin matrix protein 1 (*Dmp1*) and features high *Fgf-23*.⁹ The mouse homolog is the *Dmp1* knock-out (KO) mouse.^{9,10} Periodontal breakdown and altered cementum are reported in *Dmp1* KO mice.¹¹

Cementogenesis is sensitive to altered regulation of local P_i and pyrophosphate (PP_i); i.e., loss of tissue non-specific alkaline phosphatase (TNAP)^{12,13} in mice and humans, and lack of progressive ankylosis protein (ANK) and plasma membrane glycoprotein 1/nucleotide pyrophosphatase phosphodiesterase 1 (PC1/NPP1) in mice¹⁴ results in decreased and increased cementum, respectively. Dentin seemed to develop normally, whereas acellular cementum was profoundly affected in these cases. These models suggest local changes in P_i /PP_i affect cementum development and prompted studies of tooth root formation under conditions of altered systemic P_i . We hypothesized that acellular cementum in the *Hyp* mouse would be similar to that observed in teeth obtained from the TNAP mutant mouse, i.e., limited or absent.¹² We report, as expected, defective dentin mineralization, now quantified and, for the first time, a cementum phenotype (decreased formation of acellular cementum), as well as partial correction of the dentin mineralization defect over time in teeth from *Hyp* mice.

MATERIALS AND METHODS

Animals

Hyp mouse heterozygote breeding pairs were provided by Dr. Beate Lanske, Department of Developmental Biology, Harvard School of Dental Medicine, Boston, Massachusetts. *Hyp* males and females and wild-type (WT) littermates were sacrificed at 23, 27, 33, 45, and 96 days postcoital (dpc); birth was day 19. Animal studies were approved by the University of Washington Institutional Animal Care and Use Committee.

Histology

Mandibles were dissected, fixed in Bouin's fixative overnight, and stored in 70% ethanol. Tissues from 27 dpc and later were demineralized in acetic acid, neutral buffered formalin, and sodium chloride. Tissues were processed and paraffin embedded for the preparation of 5- μ m buccal–lingual sections of the mandibular first molar. Standard hematoxylin and eosin (H&E) staining was used for histologic observation by light microscopy.¹¹ Acellular cementum, dentin, and predentin in the first molars were measured at a site 75 μ m apical to the cemento–enamel junction (CEJ), which was selected to ensure a region limited to acellular cementum.

Statistical Analyses

All tissue measurements were obtained from three samples and compared using the Student *t* test, with significance indicated by *P* < 0.05.

Immunohistochemistry

Standard immunohistochemistry methods were used to detect the bone sialoprotein (BSP) distribution in 45 dpc *Hyp* and WT mouse molar tissues.¹⁵ Rabbit anti-mouse BSP antibody

was a gift from Dr. Renny Franceschi, Department of Periodontics and Oral Medicine, University of Michigan, Ann Arbor, Michigan. Positive staining was developed using a kit[¶] per the manufacturer's instructions. Negative control samples used normal serum in place of antibody, whereas WT acellular cementum samples served as the positive control for BSP staining.

Electron Microscopy

Mandibles from 45 dpc *Hyp* and WT mice were dehydrated via graded ethanol series, mounted, and cured in room-temperature-cure epoxy.[#] Molars were ground with 1,500 grit SiC paper at mesial surfaces, revealing the molar interior. Ultrasectioning was performed using a diamond knife^{**} mounted on an ultramicrotome.^{††} Ultrathin sections were collected onto lacey carbon-coated Cu grids.^{‡‡} Transmission electron microscopy^{§§} (TEM) was performed with a tungsten filament at 100 keV.

RESULTS

Histology

H&E-stained molar tissues from WT and *Hyp* mice were examined by light microscopy at developmental stages 23 to 96 dpc. Dentin was dramatically thinner, the zone of predentin was expanded, and pulp chambers were enlarged in *Hyp* mice as early as 33 dpc (Fig. 1). This dentin phenotype in *Hyp* mouse molars is consistent with previous descriptions of advanced developmental ages.⁶ The ratio of predentin/dentin was higher in *Hyp* molars compared to WT molars at 33 and 45 dpc, indicating a mineralization lag resulting from the *Hyp* mutation (Fig. 2). This proportional difference between *Hyp* and WT molars diminished considerably by 96 dpc, although the overall width of dentin in *Hyp* mice remained thinner (Figs. 1E and 1F; Fig. 2). Also seen was a markedly expanded and osteomalacic appearance of the surrounding alveolar bone in *Hyp* mice, starting from 33 dpc (Figs. 1A through 1D), which was still apparent at 96 dpc (Figs. 1E and 1F).

In addition to widened predentin in *Hyp* mice, an uneven mineralization front in dentin was observed at 33 and 45 dpc. This pattern was most apparent at 45 dpc, where areas of the mineralization front appeared globular (Fig. 1D, black arrows), resembling what was called “globular/interglobular dentin” in previous articles.^{6–8}

This study adds new information on dentin defects, quantifying the extent of the defect and reporting a less severe phenotype with age. Histologically, no apparent differences were noted in WT versus *Hyp* acellular cementum (Fig. 1), cellular cementum (data not shown), or periodontal ligament (PDL). Based on the observation of a marked dentin phenotype at 45 dpc, this time point was selected for further analyses of cementogenesis.

Analysis of Cementum by Immunohistochemistry

Immunohistochemistry for BSP was used as an unambiguous means of visualizing cementum because BSP is a well-established marker for cementum formation, with negligible expression in dentin and PDL.¹⁵ Low magnification showed the expected BSP distribution in the cementum and alveolar bone of WT and *Hyp* mice (Figs. 3A and 3B). Higher magnification revealed that the width of BSP staining was less in *Hyp* acellular cementum (Figs. 3C and 3D);

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^{‡‡}Ted Pella, Redding, CA.

^{§§}Philips EM420, FEI, Hillsboro, OR.

i.e., the width of BSP-stained cementum measured 75 μm apically from the CEJ was 4.3 ± 0.5 μm in WT samples and 2.3 ± 0.3 μm in *Hyp* samples ($n = 4$).

TEM Analysis of Cementum and Dentin

The difference in cementum width between WT and *Hyp* mice revealed by BSP staining prompted investigation into cementum structure using TEM on non-demineralized tissues from 45 dpc mice. TEM analysis focused on acellular cementum (Figs. 4A and 4B, boxes). The difference in acellular cementum morphology in WT versus *Hyp* molars was dramatic (Figs. 4C and 4D). WT cementum was uniform in width and distinguishable from adjacent dentin as a less dense tissue with a clear demarcation at the cementum–dentin junction (Fig. 4C). In contrast, *Hyp* mouse acellular cementum appeared uneven, with discontinuous globular features (Fig. 4D). Furthermore, in *Hyp* mice, the transition zone between dentin and cementum was indistinguishable because of comparable contrast in the two tissues (Fig. 4C versus Fig. 4D). Another finding not reported previously for dentin was the uneven mineralization represented by areas of non-uniform contrast noted throughout *Hyp* dentin (Fig. 4F). More highly mineralized globular dentin was distinguishable by darker regions.

DISCUSSION

The results from this study are consistent with our hypothesis that a decrease in systemic P_i would lead to a decrease in the formation of acellular cementum. Further, we provide new data on the root dentin phenotype, including quantification of the mineralization defect (widened predentin) and the fact that the mineralization lag is largely resolved by 96 dpc. Although histologic sections did not point to abnormalities in *Hyp* mouse cementum, immunohistochemistry for BSP revealed an altered pattern of localization of BSP in *Hyp* mouse cementum compared to WT at 45 dpc. TEM examination showed that *Hyp* mouse cementum featured discontinuous mineralization and a globular appearance. The area of cellular cementum in murine teeth is not uniform, which impedes the preparation of consistent histologic samples; thus, we chose to focus on acellular cementum.

The TEM appearance of *Hyp* mouse acellular cementum is evocative of the dentin phenotypes of hypophosphatemic mice described here and elsewhere,^{6,16–18} e.g., discontinuous mineralized pockets indicating a dentin hypomineralization phenotype, which were referred to as “interglobular” and considered a hallmark for the defective dentin associated with P_i and Vit D₃ deficiency.¹⁹ These findings suggest that pathologic reductions in serum P_i and possibly alterations in other factors may have induced a developmental cementum phenotype, although not as striking as that reported from the loss of TNAP, ANK, or PC-1/NPP1 in mice. Loss of TNAP function results in disrupted and decreased cementum formation, loss of PDL attachment, and exfoliation of teeth.^{12,13,20} Mice with loss of function mutations in *Ank* or ectonucleotide pyrophosphatase phosphodiesterase 1 (*Enpp1*) genes exhibit a profound increase in cementum formation.¹⁴ These proteins regulate microenvironmental P_i/PP_i ratios, whose effects may differ from systemic P_i changes. It is unclear if the *Hyp* cementum aberration is a direct or indirect effect of serum P_i levels; other mineralization-regulating factors must be accounted for to fully understand the cause(s) of the observed cementum phenotype, which is an area of active investigation in our laboratory.

Complex interactions govern the regulation of serum P_i , making it difficult to identify proximate and compounding causes of *Hyp* mineralization defects. *Hyp* mice exhibit dramatic increases in *FGF23* in osteocytes, and *FGF23* is higher in developing ameloblasts and odontoblasts.^{21,22} Overexpression of *FGF23* was shown to be responsible for much of the pathology in the *Hyp* mouse.^{3,23} Osteocalcin, a mineralization regulator, was increased in odontoblasts,²⁴ and the expression of P_i transporter NaP_i2b was reduced in *Hyp* mice.²⁵ Small integrin-binding ligand N-linked glycoprotein family proteins, matrix extracellular

phosphoglycoprotein (MEPE), and dentin matrix protein 1 (DMP1) contain acidic serine-aspartate-rich MEPE-associated motifs (ASARM peptides), cleavage products that are mineralization inhibitors. ASARM is reported to be increased in *Hyp* mice as a result of increased cleavage of MEPE and DMP1. Increases in ASARM have been linked to low bone mineral density.²⁶ Although ASARM levels have not been measured in cementum, this peptide may be present in increased concentrations in the cementum of *Hyp* mice and may contribute to disrupted mineralization. In vitro experiments to understand the mechanisms for the observed cementum and dentin phenotypes due to local and systemic phosphate level alterations are being conducted in our laboratory.

The literature describes dentin/pulp-related disorders in patients with XLH, including increased predentin, interglobular dentin, and enlarged pulp chambers,^{17,27–29} similar to reports of the *Hyp* mouse tooth phenotype described here and previously.^{6–8,29–31} Further, periodontal involvement, including cementum abnormalities, periodontal abscesses, and loss of lamina dura indicating alveolar bone disruption, has been noted in the teeth of patients with XLH,^{17,28} and now we report a tooth root cementum phenotype in *Hyp* mice.

CONCLUSIONS

In conjunction with previous reports, our findings suggest that hypophosphatemic individuals are susceptible to periodontal disease and should be monitored carefully to detect early signs of diseased periodontium and to administer timely treatment. Administration of a high- P_i diet normalized serum P_i and rescued bone mineralization in *Hyp* mice.^{32,33} Additionally, correction of the dentin phenotype in the permanent teeth of patients with XLH was achieved by modulation of P_i and Vit D_3 ,¹⁹ suggesting that such interventions may be successful. Although further research is needed, results from ongoing studies strongly suggest that appropriate levels of P_i and PP_i and related regulatory factors are critical for the maintenance of healthy teeth as well as bones.

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Figure 1.

Developmental time course of dentin and bone defects in *Hyp* mouse molars. First molars at 33, 45, and 96 dpc in WT (A, C, and E, respectively) and *Hyp* (B, D, and F, respectively) mice. Note that in the dentin region, mineralized dentin (mD) is heavily stained, whereas predentin (pD) is lightly stained. (H&E.) Arrows indicate globular dentin (B and D). * = osteoid (D and F); P = pulp; B = alveolar bone.

Figure 2.

Developmental defects in *Hyp* mouse dentin are most severe at 45 dpc and are partially corrected in adulthood. Widths of dentin and predentin for all ages were measured at a site 75μm apical to CEJ (n = 3). For each bar, the combination of black (mineralized dentin) and white (predentin) represents the total dentin width. Means ± SD show decreased total dentin width and an increased proportion of predentin in *Hyp* versus WT mouse molars. Statistical analysis of total dentin width for all three age groups revealed a significant ($P < 0.05$) decrease in *Hyp* versus age-matched WT samples, indicating developmental defects in *Hyp* mouse dentin. A significant ($P < 0.05$) difference in the predentin/dentin ratio in *Hyp* versus WT was also revealed at 33 and 45 dpc, indicating a considerable lag in dentin mineralization in *Hyp* mice. However, the large predentin/dentin ratio in the *Hyp* mouse diminished by 96 dpc ($P = 2.2$ compared to WT), indicating some recovery in dentin mineralization in adulthood.

Figure 3.

BSP immunohistochemistry revealed thinner cementum in *Hyp* mouse molars. BSP staining was performed on 45 dpc WT (A and C) and *Hyp* (B and D) mandibular first molars. H brackets in C indicate the width of the BSP-stained region in the WT molar. H brackets of equal size are overlaid on the BSP-stained region of the *Hyp* molar in D, showing reduced cementum thickness in *Hyp* mouse molars. H brackets are placed at the same distance apically from the CEJ in C and D. C = cementum; P = pulp; D = dentin; B = alveolar bone.

Figure 4.

Cementum in *Hyp* mice features discontinuous mineralization similar to interglobular dentin. Boxes in 45 dpcWT (A) and *Hyp* (B) mandible tissues indicate regions used for TEM investigation (C through F). Note increased mandibular bone volume, which is porous (arrowheads), in *Hyp* (B) versus WT (A) mice. Cementum in the WT sample was discerned from adjacent dentin based on contrast differences (arrows, C), whereas cementum in *Hyp* mouse molars was indistinguishable from dentin, additionally featuring discontinuously mineralized regions (D). E and F are TEM images of dentin from the same sections exhibited in C and D, respectively. Regions of non-uniform mineralization in *Hyp* dentin (F) were noted, reflecting regions of higher mineralized globular dentin (*). C = cementum, D = dentin.