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ABSTRACT

Hypophosphatasia (HPP) occurs from loss-of-function mutation in the tissue-non-specific alkaline phosphatase (TNALP) gene, resulting in extracellular pyrophosphate accumulation that inhibits skeletal and dental mineralization. TNALP-null mice (*Akp2*^{-/-}) phenocopy human infantile hypophosphatasia; they develop rickets at 1 week of age, and die before being weaned, having severe skeletal and dental hypomineralization and episodes of apnea and vitamin B₆-responsive seizures. Delay and defects in dentin mineralization, together with a deficiency in acellular cementum, are characteristic. We report the prevention of these dental abnormalities in *Akp2*^{-/-} mice receiving treatment from birth with daily injections of a mineral-targeting, human TNALP (sALP-FcD₁₀). sALP-FcD₁₀ prevented hypomineralization of alveolar bone, dentin, and cementum as assessed by micro-computed tomography and histology. Osteopontin – a marker of acellular cementum – was immunolocalized along root surfaces, confirming that acellular cementum, typically missing or reduced in *Akp2*^{-/-} mice, formed normally. Our findings provide insight concerning how acellular cementum is formed on tooth surfaces to effect periodontal ligament attachment to retain teeth in their osseous alveolar sockets. Furthermore, they provide evidence that this enzyme-replacement therapy, applied early in post-natal life – where the majority of tooth root development occurs, including acellular cementum formation – could prevent the accelerated tooth loss seen in individuals with HPP.

KEY WORDS: tissue-non-specific alkaline phosphatase, tooth loss, cementum, dentin, osteopontin.

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Enzyme Replacement Therapy Prevents Dental Defects in a Model of Hypophosphatasia

INTRODUCTION

Rickets and osteomalacia, and early tooth loss, in hypophosphatasia (HPP) reflect loss-of-function mutations in the tissue-non-specific alkaline phosphatase (TNALP) gene (*ALPL*) (Millán, 2006; Whyte, 2008). In the absence of functional TNALP, one of its natural substrates – inorganic pyrophosphate (PP_i) – accumulates extracellularly (ePPi) and inhibits skeletal and dental mineralization (Fedde *et al.*, 1999). HPP is classified clinically according to age at diagnosis, and varies remarkably in severity, spanning (from most severe to mildest) perinatal, infantile, childhood, adult, and odontohypophosphatasia forms (Whyte, 2008). TNALP-null mice (*Akp2*^{-/-}) phenocopy human infantile HPP remarkably well, since the mice are born with a normally mineralized skeleton, but develop rickets at about 1 wk of age, and die before being weaned, having severe skeletal and dental hypomineralization and episodes of apnea and vitamin B₆-responsive seizures (Waymire *et al.*, 1995; Narisawa *et al.*, 1997, 2001; Fedde *et al.*, 1999). A delay or partial failure in dentin mineralization—associated with a delay in incisor eruption, a deficiency (largely absent) in the amount of acellular cementum, and altered osteopontin distribution in bone (patchy osteoid immunostaining)—characterizes *Akp2*^{-/-} mice (Beertsen *et al.*, 1999; Tesch *et al.*, 2003; van den Bos *et al.*, 2005; Millán *et al.*, 2008). We have reported that newborn *Akp2*^{-/-} mice receiving a daily high-dose (8.2 mg/kg) subcutaneous (SC) injection of a bone-targeted, recombinant, human TNALP – sALP-FcD₁₀ – grew normally and appeared in overall good health without overt skeletal or dental disease, or epilepsy (Millán *et al.*, 2008). Thus, enzyme replacement therapy (EzRT) can prevent all of the manifestations of infantile HPP in *Akp2*^{-/-} mice. Here, we show that daily administration of sALP-FcD₁₀ prevents dentin hypomineralization and restores acellular cementum otherwise absent in *Akp2*^{-/-} mice.

MATERIALS & METHODS

Akp2^{-/-} Mouse Model of Infantile Hypophosphatasia

Akp2^{-/-} mice were created by insertion of the Neo cassette into exon 6 of the mouse TNALP gene (*Akp2*) via homologous recombination to functionally inactivate the *Akp2* gene, resulting in no detectable TNALP mRNA or protein (Narisawa *et al.*, 1997). Pyridoxine supplementation briefly suppresses the

seizures in these mice, and extends their lifespan (but only until post-natal days 18-22; Waymire *et al.*, 1995; Narisawa *et al.*, 2001). Therefore, to better document tooth changes over time after EzRT, we gave all animals (breeders, nursing mothers and their pups, and weanlings) free access to modified laboratory rodent diet 5001 containing increased levels (325 ppm) of pyridoxine. To identify *Akp2*^{-/-} homozygotes at day 0 (date of birth), we used 0.5 μ L of blood obtained from toe clippings and measured serum alkaline phosphatase (ALP) activity in a total reaction volume of 25 μ L, velocity of 30 min at OD405, with 10 mM p-nitrophenyl phosphate substrate. The genotype of the animals was confirmed by PCR and/or Southern blotting, with tail DNA obtained at the time of tissue collection. Animal use and tissue collection procedures followed approved protocols from the Sanford-Burnham Medical Research Institute Animal Ethics Committee.

TNALP Preparation and Mineral Binding

The production and characterization of the bone-targeted, soluble, human form of TNALP – sALPFcD₁₀ – have been reported (Millán *et al.*, 2008). In short, to facilitate the expression and purification of recombinant human TNALP, we removed its hydrophobic C-terminal sequence that specifies GPI-anchor attachment, thereby creating a soluble secreted enzyme, and extended the coding sequence of its ectodomain with the Fc region of human IgG (γ 1 form). This latter addition allowed for rapid purification by protein A chromatography. Furthermore, to target the recombinant TNALP to bones and teeth, we added a mineral-binding deca-aspartate (polyAsp, D₁₀) sequence as an extension to the C-terminal of the Fc region. This endowed sALP-FcD₁₀ (which spontaneously forms a dimer) with 32-fold increased affinity for hydroxyapatite *in vitro* compared with unmodified, soluble TNALP (Sigma, St. Louis, MO, USA) (Millán *et al.*, 2008). Immunogold verification of sALP-FcD₁₀ binding to synthetic hydroxyapatite (courtesy of B. Fowler and D. Eanes, NIST, Bethesda, MD, USA) in a cell-free assay was performed by incubation of a slurry of hydroxyapatite crystals with sALP-FcD₁₀, followed by washing, and then protein A-gold conjugate (14 nm gold particle size) incubation, where protein A binds with high affinity to the Fc (IgG) portion of sALP-FcD₁₀. Control incubations were identical, but with omission of the sALP-FcD₁₀ incubation step. Treated crystals were deposited onto carbon-coated mesh grids and dried for subsequent examination by transmission electron microscopy.

Energy Calculation and Structure Prediction of D10 Peptide Binding to Hydroxyapatite

All simulations were performed with the RosettaSurface Monte Carlo plus-minimization structure-prediction program (Makrodimitris *et al.*, 2007). Each execution of the program folds a peptide from a fully extended conformation and results in one energy-minimized candidate solution- and adsorbed-state structure. Large structural ensembles of 10⁵ candidate solution- and adsorbed-state structures were generated, from which the 100 lowest-energy structures from each state were chosen for further analysis. A similar polyAsp sequence from osteopontin

binding to mineral has been modeled previously with this approach (Chien *et al.*, 2009).

Enzyme Replacement Therapy Protocol for *Akp2*^{-/-} Mice

sALP-FcD₁₀ was injected at a dose of 8.2 mg/kg SC daily until the animals' death at day 16; the dose was selected from a range of doses based on their effects on the skeleton (Millán *et al.*, 2008). *Akp2*^{-/-} mice were given either vehicle (n = 18) or sALP-FcD₁₀ (n = 19). Additionally, one non-treated, wild-type mouse *per litter* was examined (n = 18). The plasma ALP activity in the *Akp2*^{-/-} mice treated with 8.2 mg/kg/day for 15 days varied from values in the normal range to values 3-6 times higher than those in wild-type controls (data not shown).

Tissue Preparation

Mandibles were immersion-fixed overnight in sodium-cacodylate-buffered aldehyde solution and cut into segments containing the first molar, the underlying incisor, and the surrounding alveolar bone. For the immunostaining and microscopy analyses, some samples were decalcified in 4% EDTA at neutral pH to allow for better discrimination of tissue histology and ultrastructure of the thin layer of acellular cementum at the dentino-cemental junction. For plastic embedding, samples were dehydrated through a graded ethanol series and infiltrated in acrylic resin (LR White; London Resin Company, Berkshire, UK), followed by polymerization of the tissue-containing resin blocks at 55°C for 2 days as described previously (McKee *et al.*, 1991), while other samples were conventionally processed for paraffin embedding.

Micro-computed Tomography

Micro-computed tomography (model 1072; Skyscan, Kontich, Belgium) of non-decalcified hemi-mandibles was performed at the level of the first molar from 3 samples of each genotype. The x-ray source was operated at maximum power (80 KeV) and at 100 μ A. Images were captured with a 12-bit, cooled, charge-coupled device camera (1024 x 1024 pixels) coupled by a fiber optic taper to the scintillator. At a rotation step of 0.9°, total scanning time was 35 min for each sample, with a scan resolution of 5 μ m, after which ~300 sections (slice-to-slice distance of 16.5 μ m) were reconstructed with Skyscan tomography software (Skyscan) based on triangular surface rendering, to give a three-dimensional distribution of the calcified tissue. Appropriate imaging planes selected to show three-dimensionalized longitudinal "sections" (segments) of the first molar and cross-sections of the underlying incisor were selected from a limited number of acquired x-ray slices.

Microscopy, and Immunohistochemistry and Immunogold Staining for Osteopontin

Thin sections (1 μ m) were cut with a diamond knife from LR White acrylic resin-embedded, decalcified hemi-mandibles (bone and teeth) by means of an ultramicrotome, and glass slide-mounted sections were stained with 1% toluidine blue. Frontal

Figure 1. TNALP fusion protein and binding to mineral. **(A)** Recombinant mineral-targeting TNALP (sALP-FcD₁₀) consists of a dimer of the recombinant, human, soluble TNALP (sALP), the constant region of human IgG1 Fc domain (Fc) (orange bars indicate 2 disulfide bonds in the adjacent hinge region), and a deca-aspartate (polyAsp, D₁₀) motif. **(B)** Transmission electron micrograph showing the binding of sALP-FcD₁₀ to synthetic hydroxyapatite crystals as revealed by immunogold labeling (inset is control incubation without sALP-FcD₁₀ showing an absence of gold-particle labeling). Magnification bar equals 100 nm. **(C-E)** Multiple views of a RosettaSurface-simulated model of D₁₀ binding to a calcium-rich plane of the [100] crystallographic face of hydroxyapatite. Asterisks (panel E) indicate COO⁻ side-chains of aspartate coordinating with surface calcium (Ca).

sections through the hemi-mandibles (at the same level of the most mesial root of the first molar) provided longitudinally sectioned molar and cross-sectioned incisor (and surrounding alveolar bone) specimens for comparative histological analyses. For immunohistochemical analysis of osteopontin in paraffin-embedded tissue, sections were deparaffinized, blocked with

normal swine serum for 60 min, and incubated overnight at 22°C with goat polyclonal antibody to mouse osteopontin (R&D Systems Inc., Minneapolis, MN, USA). Washed sections were incubated for 1 hr at 22°C with biotinylated rabbit anti-goat IgG, followed by a one-hour incubation with alkaline phosphatase (ALP)-conjugated avidin. Levamisole (1 mM) was used to block endogenous ALP activity in the tissue sections. ALP activity of the immunohistochemistry reagent ALP-avidin conjugate was detected with the Vector® Red Alkaline Phosphatase Substrate Kit (Vector Laboratories Inc., Burlingame, CA, USA) according to the manufacturer's instructions. Immunogold labeling for osteopontin coupled with transmission electron microscopy was performed as described previously for normal tooth cementum (McKee *et al.*, 1996). Electron microscopy was performed with conventionally stained (uranyl acetate and lead citrate) grid-mounted 80-nm plastic sections.

RESULTS

TNALP (sALP-FcD₁₀) Binding to Hydroxyapatite

The sALP-FcD₁₀ fusion protein dimerizes during its biosynthesis – the dimer and its components are shown schematically in Fig. 1A. Direct binding of sALP-FcD₁₀ to hydroxyapatite was confirmed by immunogold labeling and transmission electron microscopy showing abundant 14-nm gold particle-protein A conjugates along crystal surfaces when the crystals were previously incubated with sALP-FcD₁₀ (Fig. 1B), but not in control incubations without sALP-FcD₁₀ (Fig. 1B, inset). RosettaSurface adsorption energy calculations (mean ± SD) showed a binding energy of $-21.3 (\pm 6.0)$ kcal/mol, which is the difference between the mean energies of the 100 lowest-energy adsorbed- and solution-state candidates for D₁₀ adsorbed to a calcium-terminated (100) crystallographic face of hydroxyapatite, thus revealing very strong binding to the crystal surfaces. Various views of one of the 10 best, lowest-energy conformers of D₁₀ bound to hydroxyapatite are shown in Figs. 1C-1E, revealing close coordination of COO⁻ side-chains from aspartates with surface (100) lattice calcium.

Micro-computed Tomography

Micro-computed tomography (Fig. 2) of the first mandibular molar through the mesial and buccal cusps of M1, and through the incisor, followed by three-dimensional reconstructions, provided images for assessment of the mineralized tissue volume and macroscopic mineralized tooth morphology of the 3 groups of 16-day-old mice examined in this study. This revealed a consistent, major reduction of mineralization in both alveolar bone and root dentin (and root analogue dentin in the incisor) in the control (vehicle alone injected) *Akp2*^{-/-} mice (Fig. 2A) – a feature not present in the EzRT *Akp2*^{-/-} mice, which showed complete mineralization of alveolar bone and dentin (Fig. 2B) similar to that of wild-type mice (Fig. 2C).

Histology and Immunohistochemistry for Osteopontin

Whereas *Akp2*^{-/-} mice receiving vehicle alone (Fig. 3A) showed the expected (Millán *et al.*, 2008) defective molar dentin mineralization and an absence of acellular cementum along the root surface, EzRT *Akp2*^{-/-} mice had an unremarkable acellular cementum layer (Fig. 3B) comparable with that seen in wild-type mice (Fig. 3C). Immunohistochemical localization of osteopontin – a known marker and major extracellular matrix component of acellular cementum – showed a localization pattern reflecting the absence (Fig. 3D) or presence (Figs. 3E, 3F) of molar acellular cementum, as described immediately above. Similar findings were observed for both molars and incisors.

Transmission Electron Microscopy and Immunogold Labeling for Osteopontin

Transmission electron microscopy validated the absence (*Akp2*^{-/-} vehicle alone, Figs. 4A, 4B) or presence (*Akp2*^{-/-} sALP-FcD₁₀-treated, Fig. 4C; and wild-type, Fig. 4D) of acellular cementum along the root surface in the 3 groups of mice. Likewise, immunogold labeling for osteopontin – used as a marker of acellular cementum matrix – showed abundant gold particles over the cementum of EzRT *Akp2*^{-/-} and wild-type mice, but not at the molar root surface, where cementum was not present in the untreated (vehicle alone) *Akp2*^{-/-} mice (Figs. 4E-4H). Similar findings were observed for both molars and incisors.

DISCUSSION

The severity of human hypophosphatasia (HPP) varies widely, depending on the mode of inheritance (autosomal-dominant or autosomal-recessive), and the structural consequences of more than 200 individual *ALPL* mutations associated with this rare inborn-error-of-metabolism (Millán, 2006; Whyte, 2008). The different clinical forms of HPP (most severe to mildest) are perinatal HPP, infantile HPP, childhood HPP, adult HPP, and odontohypophosphatasia, and range from absence of skeletal mineralization and stillbirth to loss of teeth in adult life.

Perinatal HPP manifests *in utero* and is nearly always lethal. Some neonates may survive several days, but then suffer respiratory compromise attributable to the rachitic disease of the chest. *Infantile* HPP presents before 6 months of age, with poor

Figure 2. Targeting of TNALP to mineral restores bone and tooth mineralization defects in TNALP-deficient (*Akp2*^{-/-}) mice assessed by micro-computed tomography. Coronal views of mandibles from 16-day-old mice (vehicle-injected *Akp2*^{-/-}, sALP-FcD₁₀-treated *Akp2*^{-/-} and wild-type) as a single x-ray slice (left panels), or as reconstructions of multiple slices (right panels). (A) *Akp2*^{-/-} mice injected only with vehicle show extensive regions of unmineralized root (and root analogue) dentin (asterisks), as well as surrounding hypomineralized alveolar bone. (B) sALP-FcD₁₀ EzRT treatment of *Akp2*^{-/-} mice shows complete mineralization of all incisor and molar tooth tissues, and alveolar bone, which are indistinguishable from wild-type (WT) normal tissues (C). Arrows indicate mineralized dentin and acellular combined.

Figure 3. Presence of acellular cementum in sALP-FcD₁₀-treated (EzRT) *Akp2*^{-/-} mice visualized by histology and immunohistochemistry with anti-osteopontin antibodies. (A-C) Plastic coronal sections of the first molar near the cemento-enamel junction, showing acellular cementum along the root surface in the sALP-FcD₁₀-treated *Akp2*^{-/-} mice, comparable with that seen in wild-type (WT) normal mice (arrows and insets). This acellular cementum layer is absent (asterisks) in the vehicle-injected *Akp2*^{-/-} mice. Rectangular regions marked 1 and 2 in panel A indicate sites adjacent to mineralized (Min.) and unmineralized (UnMin.) dentin, respectively. The latter is a common feature in dentin of the hypomineralization seen in these TNALP-deficient mice; the same boxed areas are also shown by transmission electron microscopy in Fig. 4. (D-F) Immunohistochemical localization of osteopontin (red, arrows and inset), as a marker for acellular cementum, confirms the histologic observations in corresponding panels A-C showing acellular cementum with sALP-FcD₁₀ injections (*Akp2*^{-/-}-treated), but an absence of a discrete immunostained layer (asterisk in inset) when the *Akp2*^{-/-} mice were treated with vehicle alone. PDL, periodontal ligament; En-S, enamel space after decalcification. Magnification bars equal 100 μ m.

feeding, inadequate weight gain, and rickets. Serial radiologic studies may reveal worsening rickets and loss of skeletal mineralization. *Childhood* HPP also has highly variable clinical expression, with rickets causing short stature and skeletal deformities. Premature loss of deciduous teeth results from aplasia, hypoplasia, or dysplasia of the dental cementum that anchors the tooth root within the surrounding alveolar bone *via* the periodontal ligament. *Adult* HPP usually presents during middle age, although frequently there is a history of rickets and/or early loss of teeth followed by good health during adolescence

and young adult life. *Odontohypophosphatasia* is diagnosed when the only clinical abnormality is dental disease. These individuals can have only mildly reduced serum ALP levels. This milder clinical form of HPP suggests that tooth development is the most sensitive developmental process dependent upon TNALP function, a notion supported by studies in mice on cementum indicating a particular sensitivity of this tissue to local phosphate and pyrophosphate levels (Nociti *et al.*, 2002; Foster *et al.*, 2006). In some cases, tooth roots may have an absence or aplasia of acellular cementum presumably responsible for early tooth exfoliation (Bruckner *et al.*, 1962; el-Labban *et al.*, 1991; Olsson *et al.*, 1966; Hu *et al.*, 2000), and enlarged pulp chambers associated with defective dentin mineralization (Beumer *et al.*, 1973; Jedrychowski and Duperon, 1979; Olsson *et al.*, 1996; Liu *et al.*, 2010).

The *Akp2*^{-/-} mice used in the present study mimic infantile HPP remarkably well (Narisawa *et al.*, 1997; Fedde *et al.*, 1999). The fact that EzRT with a mineral-targeting form of TNALP not only prevents all of the dental abnormalities in this model of severe HPP, but also treats the severe rickets in the skeleton (Millán *et al.*, 2008), clearly shows the robustness of such a potential therapeutic approach to the selective treatment of mineralized tissues in individuals with HPP. This mineralized tissue response to TNALP EzRT in the teeth (for both dentin and cementum), and in the surrounding alveolar bone, is consistent with our previous findings of improved mineralization at other skeletal sites (Millán *et al.*, 2008). Moreover, for teeth, it provides a basis for future clinical work examining how such an EzRT might lead to tooth stabilization and retention in individuals with HPP.

Osteopontin is a prominent component of bone and cementum matrix (Bronckers *et al.*, 1994; McKee and Nanci, 1995; MacNeil *et al.*, 1995; McKee *et al.*, 1996; Bosshardt *et al.*, 1998; Yamamoto *et al.*, 2007), where, among other functions, it regulates mineralization by binding to apatite crystals in the matrix to inhibit crystal growth (Addison *et al.*, 2007). Tesch *et al.* (2003) detected an abnormal distribution pattern of osteopontin within the bone matrix of *Akp2*-null mice. While in wild-type mice osteopontin was predominantly observed in cement lines and in the mineralized bone compartment (McKee *et al.*, 1993;

McKee and Nanci, 1996), in *Akp2*-null mice osteopontin was also found throughout the non-mineralized matrix (Tesch *et al.*, 2003). Subsequently, additional work demonstrated that osteopontin levels in the *Akp2*-null mice (Harmey *et al.*, 2004, 2006) and in mouse bone cell cultures (Addison *et al.*, 2007) increase with elevated ePPI concentrations, and that normalization of osteopontin concentrations. As reported (Millán *et al.*, 2008), *Akp2*^{-/-} mice receiving EzRT have normal plasma levels of PPI, consistent with the prevention of skeletal disease. The relevance of PPI to tooth cementum mineralization is supported by our demonstration of high levels of ePPI-producing ectonucleotide pyrophosphatase phosphodiesterase (ENPP1; van den Bos *et al.*, 2005) in human periodontal ligament immediately adjacent to the cementum. Here, we show an absence of osteopontin at the tooth root surface in untreated HPP (*Akp2*^{-/-}) mice at the anatomical site normally occupied by acellular cementum, and we document normal osteopontin levels upon normalizing ePPI concentrations in the EzRT *Akp2*^{-/-} mice. Thus, supplying TNALP by EzRT to mineralized tissues appears to facilitate cleavage of inhibitory PPI, resulting in additional tooth and bone mineralization and osteopontin accumulation at tooth root surfaces to form a mineralized layer of acellular cementum.

In summary and conclusion, mice lacking the tissue-non-specific isoenzyme of alkaline phosphatase (TNALP) are an excellent model for human infantile HPP. Daily SC injection of a mineral-targeting, recombinant form of human TNALP corrects mineralization of bones and teeth and mineralized acellular cementum deposition on tooth root surfaces. Our findings provide insight concerning how acellular cementum is formed on tooth surfaces to effect periodontal ligament attachment to retain teeth in their osseous alveolar sockets. Furthermore, they provide evidence that this EzRT, applied early in post-natal life – when the majority of tooth root development occurs, including acellular cementum formation – could prevent the accelerated deciduous tooth loss seen in individuals with HPP.

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Figure 4. Presence of acellular cementum in sALP-FcD₁₀-treated (EzRT) *Akp2*^{-/-} mice visualized by transmission electron microscopy and immunogold labeling for osteopontin. (A-D) Electron micrographs showing the absence of acellular cementum in *Akp2*^{-/-} mice injected only with vehicle (panels A,B), and its presence with sALP-FcD₁₀ injections (panel C), which is essentially indistinguishable from that of wild-type (WT) mice (panel D). (E-F) Immunogold labeling for osteopontin (black particles) in acellular cementum after the sALP-FcD₁₀ injections (panel G), similar to WT cementum (panel H), but unlike the absence of gold particle labeling and lack of cementum in the *Akp2*^{-/-} mice injected with vehicle alone (panels E, H). Panels numbered 1 and 2 (in panels A, B, E, and F) correspond to the boxed areas similarly identified in Fig. 3. Magnification bars equal 200 nm. PDL, periodontal ligament; Min. Dentin, mineralized dentin.

Inc., Montreal, QC, Canada. J.L. Millán, J.J. Gray and D.L. Masica have been, or are currently, consultants for Enobia Pharma Inc., and M.D. McKee and M.P. Whyte are consultants and receive research funds from Enobia Pharma. A preliminary report of this work was presented at the 2008 annual meeting of the American Society for Bone and Mineral Research held in Montreal from September 12-16.

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