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ORIGINAL ARTICLE

Dentin structure in familial hypophosphatemic rickets: benefits of vitamin D and phosphate treatment

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OBJECTIVE: To evaluate the outcome of 1-(OH) vitamin D and oral phosphate treatment on dentin structure in patients with familial hypophosphatemic rickets, and expression of SIBLINGs (a family of non-collagenous proteins involved in dentinogenesis) and osteocalcin.

PATIENTS AND METHODS: Seven patients with familial hypophosphatemic rickets (age 3–16 years) were studied before or during treatment. Deciduous and permanent teeth were prepared for scanning electron microscopy (SEM) analysis and immunohistochemistry.

RESULTS: Untreated or inadequately treated patients had necrotic teeth with impaired dentin mineralization including unmerged calcospherites and accumulation of non-collagenous proteins in wide interglobular spaces. Most of the primary incisors analyzed displayed fissures linking enamel subsurface to pulp horn. These elements may explain the bacterial penetration and dental abscesses despite the absence of carious lesions. Well-treated patients had healthy teeth with good dentin mineralization and little evidence of calcospherites.

CONCLUSION: Treatment of hypophosphatemic children with 1-(OH) vitamin D and oral phosphate insures good dentin development and mineralization, and prevents clinical anomalies such as the dental necrosis classically associated with the disease. Starting treatment during early childhood and good adherence to the therapy are mandatory to observe these beneficial effects.
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Keywords: hypophosphatemic rickets; PHEX gene; dentin mineralization; 1-hydroxy-vitamin D treatment; interglobular spaces; non-collagenous proteins

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Familial hypophosphatemic rickets, the most common cause of inherited rickets, is associated with severe growth retardation, impaired osseous mineralization causing rickets and osteomalacia, kidney phosphate wasting and hypophosphatemia, and low circulating 1,25 dihydroxyvitamin D concentrations. X-linked dominant transmission is usual, and mutations in the PHEX gene are the main cause of the X-linked forms (HYP Consortium, 1995; Rowe *et al*, 1997; Nesbitt *et al*, 1999). However, autosomal dominant or recessive inheritances have also been reported, the autosomal dominant form being associated with mutations in the gene encoding FGF23 which lies on 12p13. Accumulating evidence suggests that FGF23 is a potent hypophosphatemic factor acting phosphatonin (Jonsson *et al*, 2003; Rowe, 2004).

Since the 1970s, 1-hydroxylated forms of vitamin D have progressively replaced vitamin D itself for the treatment of hypophosphatemic rickets, along with oral phosphate. This treatment results in better growth and a correction of bone deformities provided that it takes place early in childhood and is carefully monitored until completion of growth (Balsan and Tieder, 1990; Friedman *et al*, 1993; Makitie *et al*, 2003; Vaisbich and Koch, 2006). We previously reported its beneficial effect on the clinical dental status of children with the disorder and confirmed the critical importance of early onset of treatment to obtain the best correction of the growth, dental, and skeletal defects (Chaussain-Miller *et al*, 2003). However, even with early treatment and compliant patients, we continue to observe some remaining defects on X-ray examination of the teeth, including enlarged pulp chambers and prominent pulp horns. We suggested that these defects might result from early exposure of odontoblasts to hypophosphatemia, before the onset of treatment, and/or from intrinsic cell disturbances linked to the causative gene.

We now performed scanning electron microscopy (SEM) analysis and immuno-histological studies of deciduous and permanent teeth from treated and untreated patients with hypophosphatemic rickets to

test the effect of 1-hydroxylated forms of vitamin D and of phosphates on dentin structure and the expression of major proteins involved in dentinogenesis, namely dentin matrix protein-1 (DMP-1), dentin sialoprotein (DSP), osteopontin (OPN), bone sialoprotein (BSP), matrix extracellular phosphoglycoprotein (MEPE), and osteocalcin.

Patients and methods

Patients and specimens

Teeth from seven hypophosphatemic and seven control children were studied (Table 1). All seven patients had typical signs of familial hypophosphatemic rickets. All but patient 3 were offspring of hypophosphatemic mothers, and patients 6 and 7 were siblings. Sequencing of the PHEX gene could be performed in patients 2 and 5 and confirmed the presence of mutations. Three children (patients 1–3) were first seen at the age of 3 years, before the onset of treatment. Patient 4 was first seen at the age of 16 with necrotic permanent teeth, despite a theoretical 13 year treatment with 1-hydroxy-vitamin D and oral phosphate. However, the persistence of severe growth retardation and bone deformities suggest poor compliance with therapy and/or insufficient dosages of the vitamin D and phosphate treatment. Patients 5–7 were aged 13–16 years at the time of teeth examination. They had been followed in our department since early childhood (0.25–5 years) and treated with 1-(OH) vitamin D_3 (Unalfa^R; Leo, Ballerup, Denmark) and oral phosphate (divided into three to four daily doses). Compliance had been excellent for the girls, but more irregular during puberty for the boy.

Five deciduous and one permanent tooth had been extracted because of recurrent abscesses in the three younger patients and in patient 4, while one deciduous and two permanent teeth had been extracted for orthodontic reasons in patients 5–7. Deciduous and permanent teeth from healthy age-matched controls extracted for orthodontic reasons were also included. All teeth were obtained with the informed consent of the parents and children, and the procedure was approved by our local committee on human research. Teeth were collected immediately after extraction and fixed for 7 days in a 4% paraformaldehyde solution buffered at pH 7.3 by phosphate-buffered saline (PBS). Teeth were sectioned longitudinally under water-cooling and stored at 4°C.

SEM analysis

The first halves of the sectioned teeth were prepared for SEM analysis. Carbon or gold sputter-coated surfaces were observed under a scanning electron microscope (JEOL 30B SEM, Tokyo, Japan), equipped with an electron microprobe for X-ray microanalysis (EDAX, Ametek, Mahwah, NJ, USA).

Immunohistochemistry

The other half of the teeth was demineralized with acetic acid (0.5 M) in a solution of 0.85% NaCl and 4% paraformaldehyde (D'Souza *et al*, 1992) and embedded in Paraplast® (Oxford Labware, St Louis, MO, USA). Seven-micrometer-thick sections were prepared. Tissue sections were dewaxed and rehydrated. Endogenous peroxidases were blocked by incubation of the sections in 0.3% hydrogen peroxide in methanol at room temperature for 30 min. After extended rinsing in PBS, background activity was blocked at room temperature for 60 min in 1% bovine serum albumin (BSA) in PBS. We used five polyclonal antibodies raised against

Table 1 Clinical and biologic characteristics of the hypophosphatemic patients at the time of tooth collection

Patient	*1*	*2*	*3*	*4*	*5*	*6*	*7*
Gender	M	M	F	F	F	F	M
Age[a] (years)	3	3	3	16	13	14	16
Treatment	0	0	0	±	+	+	+
Age at onset (years)				3	0.25	3	5
Duration (years)				13	13	11	11
Unalfa (μg day^{-1})				0.4–1.5	1–2.5	1.25–1.5	1.5–2.5
Phosphates (mg kg^{-1} day^{-1})				20–50	45–50	30–60	25–50
Compliancy				Poor	Good	Good	Good[b]
Signs of rickets							
Leg bowing	+ + +	+ + + +	+ +	+ + + +	0	0	+
Height (s.d.)	−3	−2	−2	−3.7	−1	−1.5	−1.5
Serum P (mmol l^{-1})	0.69	0.90	0.69	0.59	0.68	0.69	0.66
Serum ALP (IU l^{-1})	498[d]	1263[e]	1027[e]	123[f]	225[g]	269[g]	330[g]
Dental status							
DMFT or DFT	5	4	1	11	0	2	0
Collected tooth	51,61,74	51	61	31	85	24	48
Type[c]	D	D	D	P	D	P	P
Pulp status	Necrotic	Necrotic	Necrotic	Necrotic	Healthy	Healthy	Healthy

[a]Age at the time of tooth collection.
[b]Compliance with treatment of this adolescent was good before puberty and less satisfactory after.
[c]Type of collected tooth, deciduous (D) or permanent (P).
Height is given in s.d. as regards reference curves for sex and age-matched healthy children. All serum phosphate concentrations were below normal. Serum alkaline phosphatase activities above normal range are underlined. Normal ranges (IU l^{-1}) were: [d] < 390 ; [e] < 720; [f] < 230; [g] < 300.

SIBLINGs. The anti-DSP (LF 153), anti-DMP-1 (LF 143), anti-BSP (LF 100), and anti-OPN (LF 123) antibodies were generous gifts from Larry Fisher, NIDCR, USA (Fisher and Fedarko, 2003). The anti-MEPE antibody was directed toward a synthetic peptide fragment of MEPE containing the RGD and SGDG sequence, a gift from Acologix (Emeryville, CA, USA). We also used an anti-osteocalcin antibody (OC; Abcam, Cambridge, MA, USA). All antibodies were used at 1/100 dilution. Sections were treated in a moist chamber at room temperature for 90 min and further incubated, after PBS rinsing, for 2 h with a swine anti-rabbit immunoglobulin-G peroxidase conjugate (Dako, Glostrup, Denmark), as secondary antibody. Peroxidase labeling was revealed for 15 min in a dark chamber using 3-3′ diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO, USA) with hydrogen peroxide in buffered solution (PBS). Sections were further rinsed with tap water. To exclude nonspecific binding, controls were carried out by omitting the primary antibody.

Results

Table 1 gives clinical and biological characteristics of the seven patients with hypophosphatemic rickets. The three children seen before the onset of treatment (patients 1–3) had moderate to severe leg deformities, a clear growth delay (−2/−3 S.D.), and a 30–75% elevation of their serum alkaline phosphatase activity over the upper range of normal values for their age. They also had dental problems requiring the extraction of necrotic teeth (Figure 1). Patient 4 had been treated since early childhood (3 years old) but regular survey had not been possible during the treatment, as the child had left France. At 16 years of age, the girl had severe bowing of the legs, severe growth retardation (−3.7 S.D.), and dental abscesses. In contrast, the three treated patients followed since early childhood in our department (patients 5–7) had no or slight bone deformities, slight growth delay, a satisfactory dental status [decayed missing filled teeth (DMFT)/decayed filled teeth (dft): 0–2], and normal or slightly elevated serum alkaline phosphatase activities.

SEM examination

Teeth from all patients (Figures 2a–c and 3) showed normal enamel and mantle dentin (the 30 μm thick outer layer located beneath the dentino-enamel junction) when compared with healthy control teeth (Figure 2d).

By contrast, the circumpulpal dentin displayed severe alterations in deciduous teeth from untreated patients 1–3 (Figures 2a and 3) and in the permanent tooth from the insufficiently treated patient 4 (data not shown). Large interglobular spaces were observed between unmerged calcospherites (Figures 2a and 3), while continuous dentin tubules regularly crossed a homogeneous dentin in control teeth (Figure 2a).

Furthermore, fissures were observed in most deciduous incisors, extending from the pulp horn up to the dentino-enamel junction (Figure 3). Such fissures may have facilitated bacterial penetration and pulp infection despite the lack of carious decay or trauma, a phenomenon that has been reported previously (Chaussain-Miller *et al*, 2003). Using X-ray microanalysis, no mineralization was detected in the interglobular spaces of the primary dentin, whereas it was close to controls in calcospherites (patients: P 35.2%, Ca 64.8%, ratio Ca/P: 1.84; control primary dentin: P 39.7%, Ca 60.2%, ratio Ca/P: 1.51).

The results were different in both the vital deciduous and the permanent teeth from the treated patients (patients 5–7). Indeed, calcospherites were only detected in the outer part of the circumpulpal dentin while the inner part appeared normal (Figure 2b,c). X-ray microanalysis in permanent teeth from treated patients showed normal mineralization of the dentin (patients: inner part: P 36.4%, Ca 63.6%, ratio Ca/P: 1.74/outer part: P 39.1%, Ca 60.9%, ratio Ca/P: 1.55; control permanent dentin: P 36.8%, Ca 63.2% ratio Ca/P: 1.71).

Immunostaining

The presence of dentin proteins recognizing polyclonal antibodies directed against SIBLINGs (DMP-1, DSP, OPN, BSP, and MEPE) and OC was searched for in deciduous teeth from an untreated infant (patient 1), in permanent teeth from two treated adolescents (patients 6 and 7), and in age-matched controls (Figures 4–6).

In control teeth, labeling for DMP-1 (Figure 4a), DSP, OPN, and BSP (data not shown) were located in the odontoblast/predentin border and, for DMP-1, in the inner third of circumpulpal dentin, associated with the tubular and peritubular dentin. In contrast, immunostaining for DMP-1 (Figure 4b), DSP, OPN, and BSP (data not shown), were only observed in the interglobular spaces in the dentin from the untreated patient. For the treated adolescents, it paralleled the ultrastructure heterogeneity observed by SEM analysis (Figure 2c) in the dentin (Figure 4c–f), with a normal

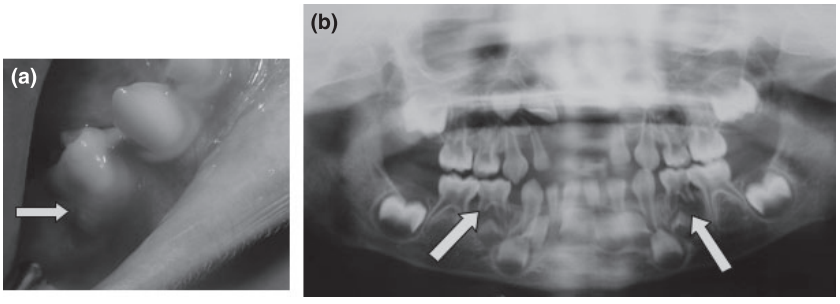

Figure 1 Clinical view (**a**) and orthopantomogram radiograph (**b**) from patient 1. (**a**) The white arrow indicates the presence of a recurrent abscess on a left deciduous molar (74). (**b**) Bone resorption is observed around or beneath the roots of the deciduous molars (74, 84 – see white arrow). All molars show prominent pulp horns extending up to the dentino-enamel junction

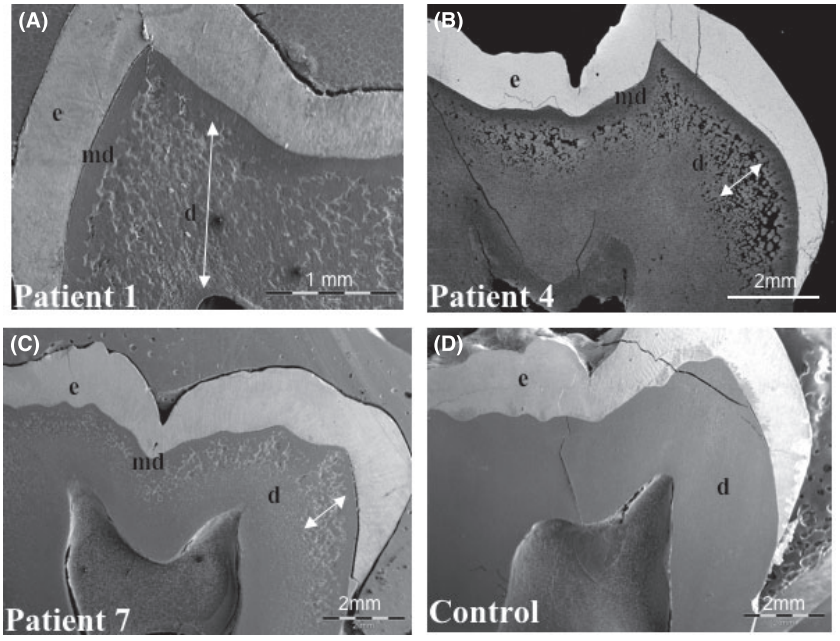

Figure 2 Crowns of molars observed by SEM from (A) a non-treated hypophosphatemic patient (74, patient 1), (B) a treated hypophosphatemic patient (85, patient 4), (C) a treated hypophosphatemic patient (48, patient 7), and (D) control patient (48). Enamel (e) and the mantle dentin (md) are similar for the patients. Calcospherites are observed in all the dentin (d) for patient 1 (A) and in the outer part for patients 4 and 7 (B and C) as shown by the white arrows

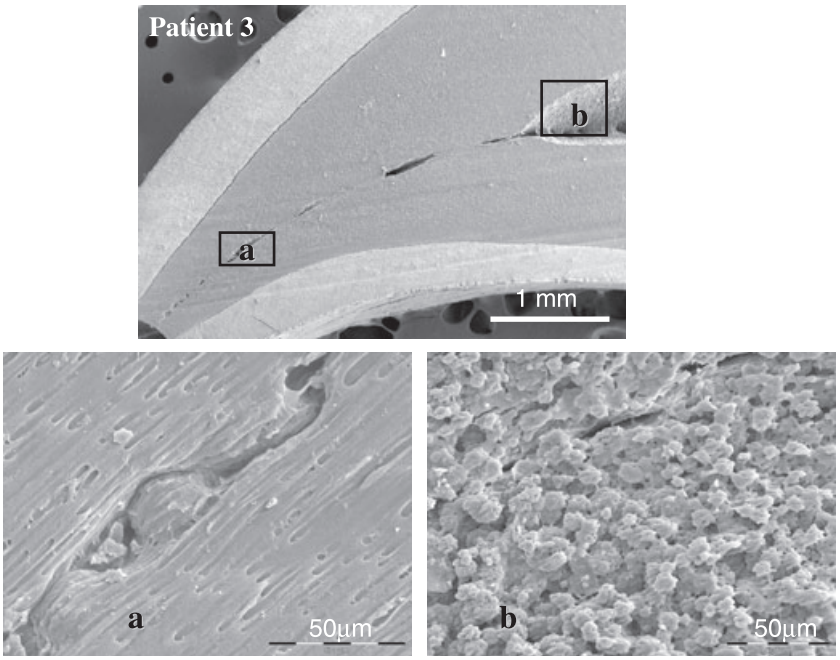

Figure 3 SEM examination of a necrotic incisor from patient 3. A large fissure closely associated with the calcospherites and extending from the pulp horn up to the dentino-enamel junction is observed (a). Calcospherites are present in the inner part of the dentin (b)

distribution in the inner part of the circumpulpal dentin (Figure 4c,e), while the outer part showed accumulation of DMP-1 (Figure 4d,f), DSP, OPN, and BSP (data not shown) in the interglobular spaces, similar to that observed in the untreated patients.

For MEPE, the control patients as well as patients 6 and 7 showed a similar and weak labeling at the predentin border, slightly above background (Figure 5a–c). This labeling was only observed in the interglobular spaces of the dentin of untreated patient 1 (data not shown) and of the calcospherite area in the dentin of patient 7 (Figure 5d).

Finally, labeling for osteocalcin was located in the odontoblast/predentin border in permanent control and treated dentins (Figure 6a,c) and was not above background in the outer area of permanent treated dentin (Figure 6d). In contrast, it accumulated in the interglobular spaces in primary hypophosphatemic dentin of the untreated patient (Figure 6b).

Discussion

The SEM and immunostaining analyses indicate that familial hypophosphatemic rickets mainly affects the

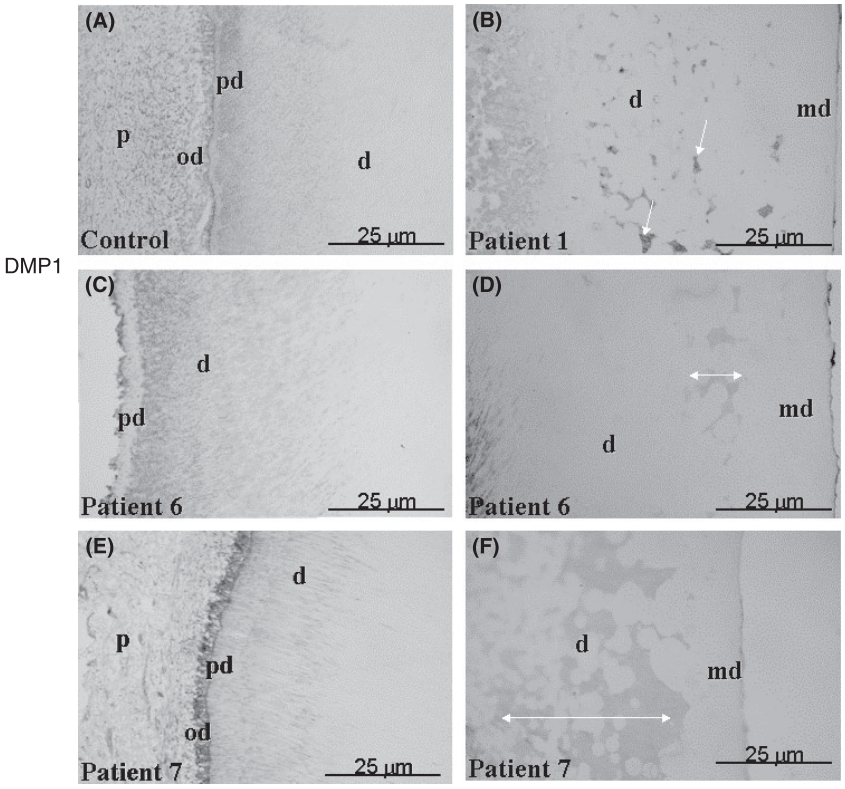

Figure 4 Indirect immunodetection of DMP-1 in dentin.
A: control patient; B: hypophosphatemic primary dentin (patient 1); C–F: hypophosphatemic permanent dentin (patient 6: C, D and patient 7: E, F). For the treated patients (C, E), odontoblasts, predentin and the inner part of dentin do not display any difference with the control (A). DMP-1 is mainly located in the interglobular spaces in primary dentin (B) and in calcospheritic areas of permanent dentin of treated patients as shown by the white arrows (D, F).
p, pulp; od, odontoblast; d, dentin; md, mantle dentin

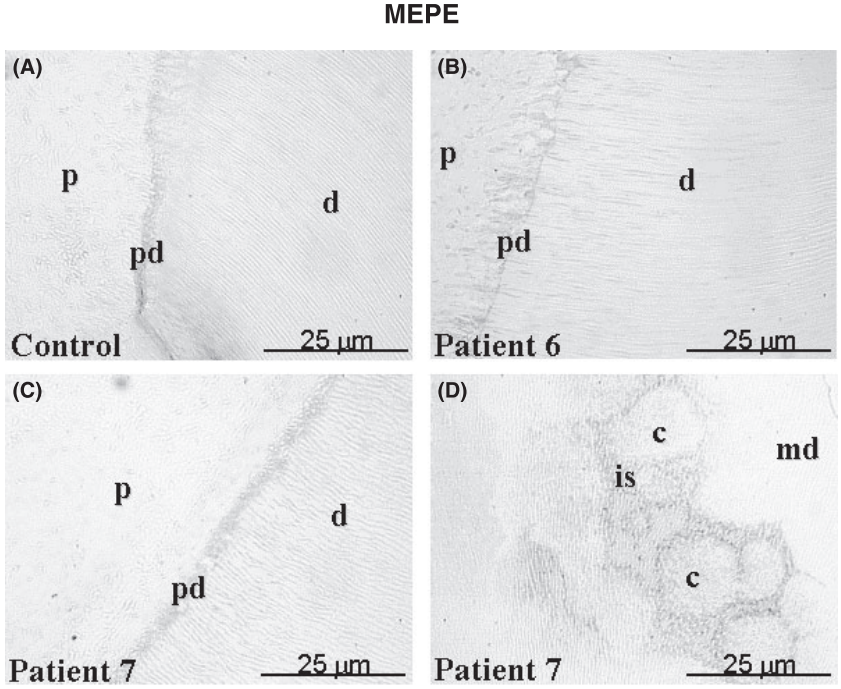

Figure 5 Indirect immunodetection of MEPE in permanent dentin.
A: control dentin, the labeling is weak, slightly above background. B–D: hypophosphatemic dentin (patient 6: B and patient 7: C, D). No labeling is seen for patient 6, whereas patient 7 displays in the calcospheritic area (outer part of dentin) a dense immunolabeling of MEPE in the interglobular spaces (D).
p, pulp; pd, predentin; od, odontoblast; d, dentin; md, mantle dentin; c, calcospherite; is, interglobular space

circumpulpal dentin of the tooth, limiting growth and fusion of calcospherites, and resulting in a porous dentin prone to bacterial invasion. This observation may explain the spontaneous tooth necrosis and is similar to previously reported findings (Seow *et al*, 1995; Goodman *et al*, 1998; Murayama *et al*, 2000; Goldberg *et al*, 2002). Furthermore, we observed in primary teeth long fissures extending upward from the pulp to the dentino-enamel junction that may have facilitated spontaneous necrosis of the teeth. These fissures may result from an impaired signaling as a consequence of the disease, which creates a lack of fusion between

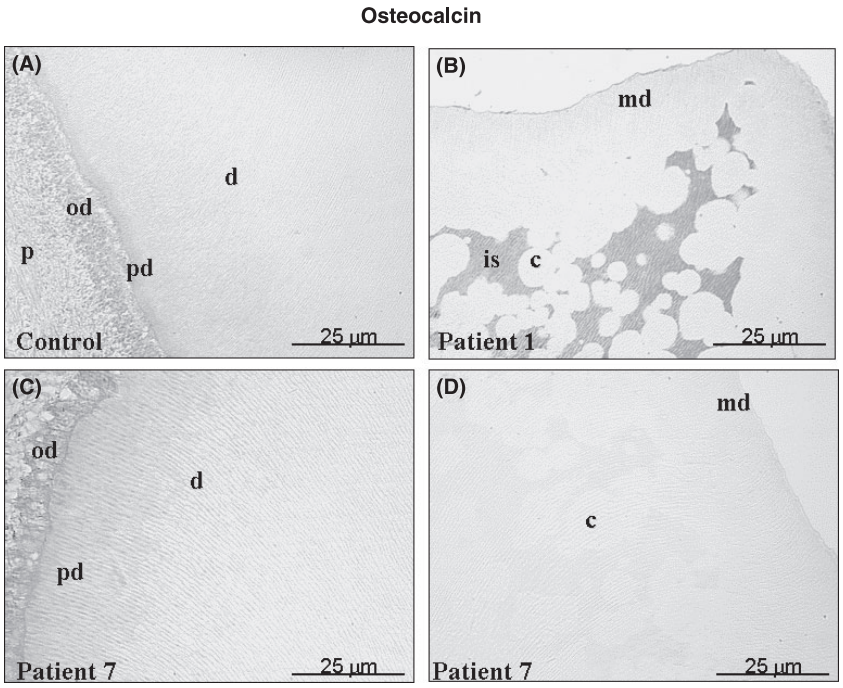

Figure 6 Indirect immunodetection of osteocalcin in dentin.
A: control patient; B: hypophosphatemic primary dentin (patient 1); C, D: hypophosphatemic permanent dentin (patient 7). For this treated patient (C), odontoblasts, predentin, and the inner part of the dentin display a pattern of labeling similar to that observed in the control (A). Osteocalcin accumulates in the interglobular spaces in the primary dentin (B) and is not above the background in the outer area of the permanent dentin for patient 7 (D).
p, pulp; od, odontoblast; pd, predentin; d, dentin; md, mantle dentin; c, calcospherite; is, interglobular space

developmental modules during tooth formation (Jernvall and Jung, 2000), by analogy with what has previously been reported in mouse models such as the Tabby incisor (Miard *et al*, 1999). Alternatively, these fissures could be cracks induced by occlusal pressure on a weaker tooth because of the altered resilience of the hypomineralized circumpulpal dentin. Indeed, the mantle dentin and the superficial part of the circumpulpal dentin constitute a layer of 250 μm essential to the biomechanical resistance of the tooth to pressure (Wang and Weiner, 1998).

The precise pathogenesis of the dental alterations observed in hypophosphatemic rickets is not yet completely understood, as both local factors and disease-related metabolic alterations, especially hypophosphatemia and 1,25-(OH)2D deficiency, may be at work. To help resolve this dilemma, we analyzed the ultrastructure and protein distribution of dentin in teeth from patients with familial hypophosphatemic rickets and compared the results obtained in untreated and treated patients.

The role of non-collagenous proteins in bone and tooth mineralization is still a matter of debate as these proteins may be nucleators or inhibitors of matrix mineralization according to the context and the experimental model used (Hunter and Goldberg, 1994; Narayanan *et al*, 2003; Qin *et al*, 2004). This is especially the case for osteocalcin (Ducy *et al*, 1996; Bronckers *et al*, 1998; Onishi *et al*, 2005). In the case of hypophosphatemic rickets, osteocalcin, BSP, vitronectin, and DMP1 have been suggested to be downregulated (Rowe *et al*, 2004). However, recent *in vitro* data have shown normal expression of osteocalcin and DMP1 in *Hyp* mice-derived bone marrow stromal cells (Liu *et al*, 2005). Moreover, *Hyp* mice odontoblasts even showed increased expression of osteocalcin with normal expression of other proteins (OPN, type 1 collagen, DMP1) during crown calcification (Onishi *et al*, 2005). However, depending on which publication one refers to, the dentin in *Hyp* mice either exhibits (Abe *et al*, 1989) or does not exhibit the structural defects seen in the human disorder (Onishi *et al*, 2005), casting doubt on the usefulness of this model with respect to dental tissues. The present study shows a marked heterogeneity in the ECM protein and mineral distribution in the dentin of untreated patients. High levels of protein expression (SIBLINGs, osteocalcin) and deficient mineralization were found in the interglobular spaces, while calcospherite areas showed close to normal mineralization with no protein expression.

Another protein, MEPE, may be upregulated in hypophosphatemic rickets, and its ASARM peptide, the cleaved C-terminal motif of MEPE, may act both as a local inhibitor of mineralization and as a phosphatonin acting directly on phosphate reabsorption at the kidney level (Rowe *et al*, 2004, 2005). In normal physiology, MEPE would be protected from cleavage by ECM proteases/cathepsin B via a non-proteolytic interaction with PHEX. In hypophosphatemic rickets, because of the mutation of PHEX, this protection may be lost, leading to increased cleavage of MEPE and to uncontrolled liberation of ASARM peptides (Rowe *et al*, 2005). This would explain hypophosphatemia and the local defects of bone/dentin associated with the disease, inasmuch as the circulating levels of the MEPE ASARM peptide are increased in hypophosphatemic patients and *Hyp* mice (Bresler *et al*, 2004). In agreement with this hypothesis, cultured bone marrow cells from MEPE-deficient *Hyp* mice show a better mineralization capacity than cells derived from *Hyp* mice, suggesting that MEPE may regulate local mineralization. However, these MEPE-deficient *Hyp* mice are still

hypophosphatemic and retain bone mineralization defects, minimizing therefore the role of MEPE in the disease, and particularly its possible function as a phosphatonin (Liu *et al*, 2005). The present study in untreated hypophosphatemic patients did not show a difference in expression and distribution within the dentin of MEPE and the other tested proteins.

As reported earlier, the treatment associating 1-hydroxylated forms of vitamin D and phosphates has a striking beneficial effect on the dental status of the patients (Chaussain-Miller *et al*, 2003). However, nothing on the main target of the disease in teeth, e.g., dentin, has been reported in humans. In the present study, we show a normal ultrastructure and ECM distribution in the inner part of the circumpulpal dentin in well-treated patients, for both primary and permanent dentin. The remaining areas of hypomineralized calcospherites observed in the outer part of the dentin may correspond to the dentinogenesis that occurred *in utero* as the children's mothers were themselves hypophosphatemic, or during the first months of life, before the onset of treatment, or during a period of poor adherence to treatment. Hence, correction of hypophosphatemia and 1-(OH) vitamin D deficiency allows normal growth and fusion of calcospherites as well as normal organization of ECM proteins in the dentin, favoring normal dentin mineralization.

These data suggest that the abnormal distribution of ECM molecules in the dentin of hypophosphatemic patients may not result from a local dysfunction directly linked to the mutation of PHEX, as proposed earlier (Qin *et al*, 2004; Rowe *et al*, 2005), but is more likely a consequence of the hypophosphatemia and 1-(OH) vitamin D deficiency. Indeed, 1-(OH) vitamin D regulates either directly or indirectly the expression of most of these proteins (Bronckers *et al*, 1998; Qin *et al*, 2004). Moreover, most of these proteins (with the exception of osteocalcin) are phosphorylated and phosphate deficiency in the bone and dentin matrix may lead to a defect in protein phosphorylation, modifying the ability to promote mineralization. Nevertheless, the effect of PHEX mutations on dentinogenesis does not exclude the possibility of other local effects of the PHEX mutation on tooth development. These as yet unidentified effects may, for example, concern the development of the pulp chamber and pulp horns; enlarged pulp chambers and prominent pulp horns have been observed even in well-treated patients (Chaussain-Miller *et al*, 2003).

In conclusion, human dentin is a good marker of the efficacy of 1-(OH) vitamin D and oral phosphate treatment of familial hypophosphatemic rickets. Early detection and treatment and good adherence to treatment provide a good dental future to the patients, limiting spontaneous dental necrosis and dental disease classically associated with familial hypophosphatemic rickets.

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