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Sphingomyelin degradation is a key factor in dentin and bone mineralization: lessons from the *fro/fro* mouse

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Introduction

Lipids play major biological roles as components of cell membranes (Vance and Vance, 1991). They are involved in cell proliferation, differentiation and survival (Cutler and Mattson, 2001). Impairment of lipid functions has direct consequences on cell functions, as demonstrated by nutritional and genetic pathologies related to lipid metabolism.

As components of the extracellular matrix (ECM) of mineralized tissues, lipids have been far less studied. Their presence is now well recognized, but their role in biological and pathological mineralizations is far from being elucidated. The total lipid content of bones has been reported to range from 1–10%. In dental tissues, the total lipid content is 0.26–0.36% in dentin, 0.014% of which are phospholipids, and 0.60% in enamel, among which 12.5% are phospholipids (0.075% of total enamel) (see for review Goldberg and Boskey, 1996). Compared with the major dentin ECM components, namely the phosphorylated proteins of the SIBLING family, lipids appear as minor components. Due to the low ratio between lipids and the total ECM molecules and to the fact that the major part of the lipid fraction is lost after routine fixation and processing for light and electron microscopy, they have been less investigated than other matrix components easier to preserve and analyze.

The chemistry and histochemistry of dentin lipids

The interest in lipids was stimulated by the finding that in dentin and enamel, about 66% of the total lipids are extracted prior to demineralization and 33% only after demineralization (Shapiro *et al.*, 1966). The lipid composition of the non-demineralized extract suggests that its composition is close to that of biological membranes, namely sphingomyelin (SM), phosphatidylcholine (PC), and phosphatidylethanolamine (PE). In dentin, some of these lipids may originate from odontoblast processes located inside dentin tubules. The lipid fraction extracted after demineralization has a different composition. Indeed, the acidic phospholipids (PLs) that are associated with the mineral phase include phosphatidyl inositol (PI), PC, PE, phosphatidyl serine (PS), phosphatidic acid (PA) and cardiolipin (CL). Presumably, there is a close association between these acidic phospholipids and the surface of the crystallites and they are released only after mineral dissolution. This finding points out that a group of acidic

phospholipids is interacting with the mineral phase, and therefore, they may be involved in dental mineralization.

For a long period of time, histochemical reports aiming to detect and localize lipids in bone and dental tissues were obtained after formol-saline fixation, hot pyridine or ethanol-benzene extraction, demineralization and gelatin embedding. After such treatments, a Sudan black B stainable material was detected at the junction between predentin and dentin, osteoid and bone, and at the limit between the forming and mineralizing enamel (Irving, 1958, 1959; Irving and Wuthier, 1968). However, the method used was questionable because after such treatment, most, if not all, lipids would have been spontaneously lost or extracted from the tissue. As a matter of fact, it was demonstrated that lipophilic proteins and not lipids are stainable by this histochemical method (Fincham *et al.*, 1972).

It is well established that during fixation and dehydration most lipids are solubilized and that translocation of the remaining lipids occurs during histological processing and tissue embedding (Goldberg and Boskey, 1996). However, we found in the literature two methods allowing preservation of phospholipids within dental tissues while avoiding gross translocation.

Malachite green in the presence of aldehyde forms insoluble complexes with cellular and extracellular lipids (MGA), and the post-fixation with osmium tetroxide reinforces the electron density of the complexes, therefore facilitating ultrastructural detection (Teichman *et al.*, 1974). The specificity of the staining is better after treatment of the section with lead citrate, whereas uranyl acetate also stains proteinaceous components, inducing additional nonspecific staining. Using spot tests, we have shown that this method preferentially stains PS and SM, and to a lesser extent PC (Vermelin *et al.*, 1994). This method allowed visualizing of a fibrillar and granular material inside the predentin, located in the intercollagenic spaces. The dentin edge at the predentin/dentin junction was not stained, and in dentin, electronlucent group of collagen fibers were surrounded by MGA-positive needle-like structures. They are reminiscent of the “crystal ghosts” described by Bonucci in the mineralized cartilage and in bone, formed by phospho-glyco-lipoproteins (Bonucci, 1987). The structures persisted after demineralization when the sections were floated over EDTA or acetic acid. The demonstration that dentin crystallites are formed or are located inside lipoprotein envelopes sheds lights on the potential role of some lipids in the mineralization processes (Goldberg and Septier, 1985, 2002). After rapid-freezing followed by freeze-substitution, a method that presumably avoids the artifacts induced by chemical fixation and dehydration, the same features were observed (Goldberg and Escaig, 1987). In the predentin, rapidly frozen MGA positive structures were thicker than after aqueous chemical fixation forming a network of liposome-like structures. The MGA positive structures were abolished by pretreatment of the samples with methanol, but not by acetone, thus confirming they are actual phospholipids (since acidic phospholipids precipitate in acetone). They resist enzyme digestion by bovine testicular hyaluronidase, but are largely reduced and even abolished by phospholipases, and therefore are distinct from the proteoglycans located in the same intercollagen areas.

The iodoplatinate (IP) method indicates a similar localization of the PLs, although the molecules that are visualized are not identical to the MGA stained components. The spot test assay indicates a major affinity of IP for SM and a strong staining is also seen for PE and PC, whereas the staining appeared weaker, as a narrow ring, around the PS dot (Vermelin *et al.*, 1994). In the predentin, IP positive granules are detected in the spaces between collagen fibrils, and again, in dentin, needle-like structures are associated to the crystallites. The transition between predentin and the dentin edge is unstained. In addition, large electron-dense amorphous areas are seen in dentin, disappearing when the sections are treated with chloroform/

methanol or phospholipase, but are still present after acetone or hyaluronidase treatment of the sections.

From our histochemical investigations, it may be concluded that some phospholipids are actually present as extracellular matrix components. In the predentin, they appear as spacefilling components between collagen fibrils, sharing this location with proteoglycans. Although the reasons for such co-distribution have not yet been clarified, interaction between decorin, low density lipoprotein (LDL) and type I collagen is reported in another biological model (Pentikainen *et al.*, 1997). Association between proteoglycans and matrix vesicles was shown in the extracellular matrix of growth plate cartilage (Wu *et al.*, 1991). The same physico-chemical interaction may also apply to the predentin situation. Water desorption is due to the self-association of hydrophobic molecules that combine and form an appropriate volume where ions concentrate. These events contribute to the pre-patterning of an ordered precipitation. This cascade is suggested by the Glimcher's model of enamel formation and mineralization (Glimcher, 1979), and provides an acceptable theory for a potential role of phosphorylated proteins in enamel mineralization, a working hypothesis that may be expanded to acidic phospholipids (Vogel and Boyan-Salyers, 1976).

The origin of dentin phospholipids

Until recently, the origin of dentin ECM phospholipids was not clarified. It was assumed that they were synthesized by the odontoblasts and further secreted as matrix components. To investigate if this assumption was true, we intravenously injected (^3H) choline into rats and studied by radioautography how the radiolabeled precursor was incorporated into the cells, and released into the predentin and dentin. To avoid loss and translocation, the rats were perfused with MGA, the incisors dissected out and segments were post-fixed with osmium tetroxide. Surprisingly, after 30 minutes, no radiolabeling was detectable within the odontoblasts, but a significant labeling over background, was already located over the dentin compartment. After 1h, the situation was identical, and it took two hours before the cells began to be significantly radiolabeled (Goldberg *et al.*, 1999). Hence, dentin PC could not arise from a synthesis by the odontoblasts, followed by exocytosis, as is the case for proteins. As an alternative pathway, we hypothesized that isotopic exchanges between labeled serum lipoproteins and the outer membrane leaflet of erythrocytes that take place within 5–20 minutes, and these events immediately precede the diffusion of radiolabeled serum albumin between the odontoblasts. Radiolabeled albumin within 1h crosses the leaky intercellular junctional complex between odontoblasts, and diffuses in predentin and dentin (Kinoshita, 1979). Albumin has been recognized to be a potent lipid carrier (Veerkamp and Maatman, 1995). Altogether, this suggests that dentin phospholipids may originate from the blood serum, possibly as Low Density Lipoproteins (LDL). Rapid phospholipid interconversion and the chemical plasticity of the different forms of phospholipids as well may regulate the final phospholipid composition of the ECM.

A search for experimental evidence

These data lead us to search for examples that would substantiate the role of lipids (phospholipids) in dental mineralization. Along this line, we investigated experimental animal models involving chemically-induced lipidosis [the suramin-induced mucopolysaccharidosis and lipidosis, chloroquine-induced lipidosis], diseases induced by deficient food intake (Zinc-deficiency and/or essential fatty acid deficiency), and human genetic mutations as well (the Krabbe galactoceramide lipidosis), (see for review: Goldberg and Boskey, 1996), or targeted mutations affecting dentin and bone (the Fabry's mice, Goldberg *et al.*, 2005; Boskey *et al.*, 2006). Although slight abnormalities were detected, we did not obtain strong evidence for defective mineralization when the lipid composition is altered, and consequently the role of

phospholipids in dental mineralization was still not substantially demonstrated. At that point, our attention was directed to the study of the teeth of the *fragilitas ossium* (*fro/fro*) mouse.

The *fro/fro* mouse

The chemically induced autosomal mutation named *fragilitas ossium* is not linked to any of the collagen defects shown to be responsible of most forms of *osteogenesis imperfecta* (OI). Type I-IV constituting about 90% of OI forms are due to the mutation of genes encoding for type I procollagen, whereas type V- VII (10%) are OI lacking collagen mutations. The mutation was induced in the mouse germ line by the chemical mutagen tris (1-aziridiny) phosphine sulphide (Thiotepa®) two weeks before mating (the targeted germ cells were then spermatids). The mutant mice display a severe form of Type II and III OI (Guenet *et al.*, 1981; Sillence *et al.*, 1993), and therefore the mutation was designed as *fragilitas ossium* (*fro/fro*).

Compared to the heterozygotes mice (*+fro*) used as control, newborn homozygote mice (*fro/fro*) are of smaller sized, with brittle long bones and ribs (Muriel *et al.*, 1991). Longitudinal sections of the mandible evidence shorter incisors in the *fro/fro*, about half-length of the heterozygote (Figs 1a,b). Immunolocalization of the Proliferation Cell Nuclear Antigen (PCNA) shows a very low labelling in the forming part of the newborn *fro/fro* tooth, in contrast with the numerous labelled cells seen in the lingual part of the *+fro* incisor. The von Kossa staining confirms the reduction in number and thickness of alveolar bone trabeculae in the *fro/fro* compared with the *+fro* mouse (Figs 1c,d). Because the mineralization is impaired, the specific staining for CS/DS glycosaminoglycans is firmly enhanced using a specific (2B6) antibody in the *fro/fro*. Although decorin and biglycan display about the same staining pattern in the incisors of mutant and control mice, the staining of the mandibular bone was enhanced in the homozygote, especially with anti-biglycan. Anti-amelogenin labeling was decreased in the incisor, but enhanced in the molar, both in the epithelial ameloblast compartment and in odontoblasts. Dentin sialoprotein and osteopontin were decreased in the *fro/fro*. Despite what had been published in a previous report on long bones (Muriel *et al.*, 1991), no difference was detected for osteonectin between the *fro/fro* and *+fro* mice teeth.

Adult *fro/fro* mice recover an almost “normal” phenotype as they age. However, the closure of the lingual part in the forming zone of the incisor is delayed in the *fro/fro*, and a fissure persists between the two lingual arced portions. Why the two arches do not merge rapidly as it is the case in the control teeth has not yet been explored. In the rodent, the molar is not a continuously erupting tooth in contrast with the incisor, and as there is no turnover in dental tissues, it may be expected to reflect unequivocally the effects of the mutation. In the *fro/fro* a thick layer of cellular cementum gives the root a bulbous appearance. In addition, the pulp chamber is filled with a homogeneous well-mineralized dentin-like structure (Figs 1e,f and Figs 2a,b). The general appearance of the tooth was characteristic of that of a human type II *dentin dysplasia* (Opsahl *et al.*, 2005, Aubin *et al.* 2005).

In a previous report, it was suggested that the *fro/fro* mutation might be linked to an osteonectin (or SPARC protein) defect, (Muriel *et al.*, 1991), a gene mapping to chromosome 11. The mutation in *fro/fro* was later localized on mouse chromosome 8. A deletion was found, encompassing part of intron 8 and a major part of exon 9, including the coding sequence of this gene (Aubin *et al.*, 2005). The cause of the mutation was identified as a sphingomyelin phosphodiesterase neutral 2 enzyme (*Smpd3*) deficiency. Indeed, in the cultured fibroblasts of the *fro/fro* mouse, *Smpd3* activated by tumor necrosis factor- α (TNF- α) has an enzyme activity reduced to $12 \pm 4\%$ compared to *+fro*.

Neutral sphingomyelinases cleave sphingomyelin to produce ceramide and phosphocholine. They are activated by TNF- α and by several growth factors. Overexpression of neutral sphingomyelinase stimulates apoptosis in human aortic smooth muscle, but not in human

hepatocytes (Chatterjee, 1999). Using the TUNEL method to detect apoptotic cells, we were unable to detect any difference between the mutant and the control mice in teeth and alveolar bone. The consequences of *Smpd3* impairment on mineralized tissues are now better documented (Aubin *et al.*, 2005; Stoffel *et al.*, 2005). In addition, preliminary results obtained on adult femurs by infrared microscopic analysis (Spevak and Boskey, unpublished results) show that *fro/fro* cortical bone has lower mineral/matrix and carbonate/mineral ratio and a little bit less collagen crosslink compared to the *+/fro* mice, analogous to what is seen in bones of humans and animal models of *osteogenesis imperfecta*.

Glycosphingolipids have been identified in bone, cartilage, and in dentin (Fukaya *et al.*, 1989; Goldberg and Boskey, 1996; Goldberg and Septier, 2002). Sphingomyelinase is involved in sphingomyelin hydrolysis in osteoblast-like cells (Kozawa *et al.*, 1997). Extracellular *Smpd* amplifies BMP-4-induced osteocalcin synthesis in osteoblast-like MC3T3-E1 cells (Kozawa *et al.*, 2002). Finally, during cartilage matrix vesicle-induced mineralization, there is a progressive disappearance of sphingomyelin, presumably due to hydrolysis by sphingomyelinases (Wu *et al.*, 2002). Altogether, adding these data to what we have observed in the *fro/fro* mutation in dental tissues and in mandibular and long bone as well, we suggest that proper sphingomyelin metabolism is essential for mineralization in bone and dentin. To the best of our knowledge, the *fro/fro* mice provide the very first *in vivo* experimental evidence that sphingomyelin and moreover sphingomyelin degradation plays a crucial role in bone and dentin mineralization. In addition, this new finding suggests that it is necessary to revisit the classification of the non-collagenous forms of *dentinogenesis imperfecta* and *dentin dysplasia* (Witkop 1988; Waltimo *et al.*, 1995).

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Figure 1.

Longitudinal sections of mandibles of newborn mice 1a: *+/fro* and 1b: *fro/fro*. The incisor is shorter in the *fro/fro* mouse. The profile of the molar (m) is affected by the mutation. Fig 1c and 1d are transverse sections of the mandible of *+/fro* and *fro/fro* newborn mice. The von Kossa staining reveals that the alveolar bone of the *fro/fro* mouse is hypomineralized, with less trabeculae (arrows). Because the incisor is shorter in the *fro/fro* mouse, no incisor is seen in fig 1d when the section plan is at the level of the first molar (m). In 1a-d, bars = 100 μ m. Fig 1e is a transverse section of a young *+/fro* adult, with a large pulp (asterisk). The alveolar bone (ab) is dense. In contrast, fig 1f shows in a transverse section of an age-matched adult *fro/fro* that the pulp chamber in the molar is filled with a dentin-like tissue (asterisk). Alveolar bone (ab) looks less compact compared with the *+/fro*. Figs 1e and f: bars= 150 μ m. p: pulp; ab: alveolar bone). Bar: 1mm.

Figure 2.

Fig 2a: Higher magnification of the apical part of the molar of an adult *fro/fro* mouse. The formation of a thick layer of cellular cementum and the filling of the root pulp (p) by a dentin-like tissue in continuity with the dentin (d) constitute the dental phenotype of the mutated mouse. The periodontal ligament (pdl) and alveolar bone (ab) look normal Bar= 150 μ m. The transverse section of an adult *fro/fro* mouse observed with a scanning electron microscope (Fig 2b) displays similarities with the human type II *dentin dysplasia*. (e: enamel; d: dentin;