Lipids in Enamel and Dentin: Involvement in Mineralization

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Abstract:
In mineralized dental tissues, lipids are minor components of the extracellular matrix. About 66 percent of lipids are extracted from the mineralized enamel or dentin, and 33 per cent is removed after demineralization. Lipid composition suggests that the first extract is associated with biological membranes whereas the second extract obtained after demineralization is linked to the mineral phase. Histochemical staining was for a long period of time limited to Sudan black, but the conclusions were that this method identified proteins rather than true lipids. Two other methods (malachite green and iodoplatinate) allowed to identified lipids and phospholipids on sections of dental mineralized tissue. A series of drug-induced lipidosis were unable to provide any conclusive answer regarding the role that lipids may play in the mineralization of dental tissues. Fatty acid deprived diet and zinc-deficient food intake, or using pharmacological drugs such as chloroquine or suramin, inducing lipidosis, displayed cell alterations, but did not provide a clear-cut explanation on the lipid implication in mineralization. Similar results were obtained investigating the Fabry mice, and/or Krabbe disease, that displayed genetic lipidosis. Gene encoding the neutral sphingomyelin phosphodiesterase 2 enzyme (Smpd3) deficiency lead to a complete loss of enzymatic activity and subsequently to a lipid deficit (fragilitas ossium-fro/fro mice). This mutation provide evidence that lipids (and/or phospholipids) play a crucial role in dental and bone mineralization, but despite all the progress which were made, the actual mechanisms need still to be elucidated.

Keywords: Phospholipids, Sudan black, malachite green, iodoplatinate, gene mutation of sphingomyelin phosphodiesterase (SMPD3), fro/fro mice, enamel, dentin, bone.

Introduction
Lipids are hydrophobic organic compounds. These macromolecules include fatty acids insoluble in water, but soluble in non-polar organic solvents (acetone, chloroform, benzene, toluene). Certain components of membranes perform functions as energy-storage molecules and/or chemical messengers. Lipids may be classified into five main categories: fatty acids, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, (together with 3 additional lipids that are not found in mammalian mineralized tissues). In mineralized tissues, lipids account as minor components. However, they are present both in cellular membranes and in extracellular matrix. Lipids are structural components of membrane, acting as energy storage molecules. Lipids are also mineral-associated lipo- or phospho-proteins of enamel, dentin and alveolar bone.
Phospholipids

Phospholipids constitute a large variety of amphipathic molecules with a hydrophilic head and two hydrophobic tails. They are involved in many cellular functions. Mainly found in biological membranes, they are responsible for its visco-dynamic and more specific properties as follow (Vance & Vance, 1991). They are the cellular precursors of second messengers, inositol triphosphate and diglyceride play roles as local mediators. The liberation of these mediators is controlled by phospholipases. Their synthesis and turnover occur through various pathways including de novo synthesis and remodeling processes. The molecular species containing poly fatty acids are synthesized by de and re-acylation cycles involving phospholipases and various trans-acylation mechanisms dependent or not on the presence of ATP and CoA.

Without demineralization, about 60% acidic phospholipids could be extracted. After decalcification acidic phospholipids were extracted; except cardiolipin which could be extracted from calcified dentin (Shapiro et al., 1966; Prout et al., 1973). Mature enamel matrix contained 26 time more lipids than dentine matrix. (Odutuga & Prout 1974). (Figure 2&3)

In calcified tissues, lipids are extracellular matrix components. In cartilage, phospholipids are associated with mineral and membrane (matrix) vesicles: phosphatidylserine and alkaline phosphatase. They are involved in calcium hydroxyapatite crystals formation. Associated to Ca:Pi complex they form phospholipid:Ca:Pi complexes (CXPL). Specific membrane lipids (called proteolipids) participate in CPLX formation and HAp deposition (Boyan et al, 1989).

Lipids removed prior demineralization have a composition close to biological membranes, [sphingomyelin (SM), phosphatidylcholine (PC) and phosphatidylethanolamine (PE)]. The acidic phospholipids associated with the mineral phase include phosphatidylinositol (Pi), PC, PE, phosphatidylserine (PS), phosphatic acid (PA) and cardiolipin (CL). No difference in composition appears between cortical bone and bone marrow.

In bone, characterization of the glycosphingolipids was carried out on cortical bone and cartilage (Fukaya et al, 1989). At early stages of bone formation, or during fracture healing, matrix vesicles (MV) play crucial role.

Matrix vesicles (MV) may come from the budding of the plasma membrane. The release from MV of Ca$^{2+}$ and P1 is a result of adenosine triphosphate (ATP) complex in presence of annexin (forming a PS-Ca$^{2+}$P1 complex). During phase I, there is an increased activity of MV phosphatases including alkaline phosphatase, adenosine triphosphatase, pyrophosphatase, Ca$^{2+}$-binding compounds such as the annexin family and PS. These molecules are found near the MV membrane. The carbonic anhydrase is found inside the MV, and Ca$^{2+}$ is precipitated (converted) into an intermediate octacalcium phosphate. In phase II, the crystals penetrate through the MV membrane, and ultimately destroy the MVs and forming an initial mineralized layer. (Figures 4,5).

Once the formation is initiated, mesenchymal stem cells contribute to bone mineralization through two different mode of formation (intramembranous or endochondral bone). Mesenchymal cells differentiate directly into osteoblasts through intramembranous ossification. The mesenchymal cells may differentiate into chondrocytes and they are at the origin of endochondral ossification. The total lipids extract from human bone has an average of 2,005 mg/100 g (Dirksen & Marinetti. 1970).
In bovine predentin, the major phospholipids were phosphatidylcholine (52-56% of the total phospholipids) and ethanolamine phosphoglycerides (22-23%). The phospholipid and fatty acid composition of predentin lipids were different from that of dental pulp, with higher percentage of PS and SM (Ellingson et al., 1977).

In dentin, the staining of lipids in dentin matrix was unclear for a long time (Allred, 1968) Undecalcified ground sections treated with 0.02 per cent acetic acid was mostly unstained. The peritubular dentin alone was the only compartment stained by Soudan black, as well as the limit between the forming and maturing enamel (Irving 1958, 1959; Irwing & Wuthier, 1968). In dentin, the acidic phospholipids extracted prior demineralization took origin from the odontoblast processes located inside dentin tubules, and secretory ameloblasts Tome’s processes remnants. Cholesterol esters, triglycerides, fatty acids, cholesterol, diglycerides, monoglycerides, and various phospholipids were identified in dentin. In addition, sound dentin was found to contain inositol phosphatide, sphingomyelin, lecithin, phosphatidylethanolamine, lysocephalin, and three unidentified phosphatides that may be polyglycerol phosphatides and/or phosphatidic acids. (Dirksen, 1962; Dirksen & Marinetti, 1970; Prout et al., 1973).

Analysis carried out on root dentin demineralized with EDTA indicated that the major components were palmitate (19-25%), stearate (14-17%), and oleate (20-21%). Fatty acids were found to be 10 mg/g of enamel and 14.00 mg/100g of dentin (Prout & Shutt, 1970) Average per 100g dentin was 20.28 mg, 1.97 mg /100g for enamel and 2.005 mg/100 g for human bone.

In peritubular dentin, analysis was carried out on demineralized material, and a calcium-proteolipid-phospholipid-phosphate complex was detected. Peritubular dentin does not contain collagen but is glutamic acid rich. PC, PS, PI are present along with a mannose-rich glycan and C-4-S and C-6-S proteoglycans (Gotliv & Veis, 2007).

In enamel, 44% is removed prior to demineralization, with cholesterol esters, triglycerides and free fatty acids. Lipids extracted prior to demineralization constitute 66% of the lipids, and 33% are extracted only after demineralization. (Shapiro et al., 1966). After demineralization, the same profile was detected. (Fincham et al., 1972, 1999). In the extract, 0.60% was established as lipids, (Goldberg and Boskey, 1996). The content in enamel proteins was revealed by chemical studies (Fincham et al., 1999).

**Histochemistry of dentin & enamel lipids**

After formol-saline fixation, hot pyridine or ethanol-benzene extraction, demineralization and gelatin embedding, a stainable Sudan black material was detected at the junction between predentin and dentin, osteoid and bone, forming and maturing enamel. Although the staining was seen at the limits between unmineralized and mineralized tissues, it was concluded that lipophilic proteins and not lipids were stainable by this histochemical method (Fincham et al., 1972, 1999). In the extract, 0.60% was established as lipids, (Goldberg and Boskey, 1996). The content in enamel proteins was revealed by chemical studies (Fincham et al., 1999).

Two histochemical methods developed more recently were used successfully on dentin and enamel.

- **Malachite green form insoluble complex reinforced with osmium tetroxide, a method that preferentially stains PS, SM, and PC (Vermelin et al., 1994).** In bovine predentin the major phospholipids were phosphatidylcholine (52-56% of the total phospholipids) and to a lesser extend, ethanolamine phosphoglycerides (22-23%) (Ellingson et al., 1977). They were also identified in bone as phospho-glyco-lipoproteins (Bonucci, 1987).

The MGA positive structures were abolished by pretreatment of the samples with methanol, but not by acetone, thus confirming they are actual phospholipids. They resist enzyme digestion by bovine testicular hyaluronidase, but are abolished by phospholipases, (Goldberg & Escaig, 1987; Goldberg & Septier, 1985).

- **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 (Vermelin et al., 1994).** In the predentin, IP positive granules needle-like structures are associated with the crystallites. In addition, large electron-dense amorphous areas are seen in dentin, disappearing when the sections were treated with chioroform/methanol or phospholipase, but still present after acetone or hyaluronidase treatments of the sections.

Experimentally data using pharmacological drugs or essential fatty acids deficient diet with modified food intake evidenced that lipids or phospholipids play important role in biological mineralization. (Figures 6, 7)
• The detection of cholesterol using the fluorescent probe polyene antibiotic filipin which specifically bind to 3β hydroxysterols stained spherical particles, elongated crystals and granular or amorphous calcium deposits, but remains negative oil-red-O-stained lipids. Filipin, a probe for cholesterol, varied in density along the plasma membrane of secretory ameloblasts, and visualized membrane remnants inside the forming enamel. In dentin, organic envelopes coated the crystals, and were named “crystal-ghost” structures outlining groups of collagen fibrils (Figure 6).

• Using imidazole-osmium tetroxide fixation of rat incisors, cellular unsaturated fatty acids were stained, so that iodoplatinate visualize domains specific to the membrane, coated pits, and endocytic inclusions. Endocytic domains, rafts, and some membrane domains were stained specifically. Essential fatty acid deficient diet and/or Zinc-deficient diet for 28 days induced the following changes (Figure 7). The lipid levels in the forming portions of zinc-deficient incisors were 30–50% below control levels. They and were associated with 1) longer Tomes’ processes, 2) porosities in the forming enamel and 3) fewer malachite green-aldehyde-phospholipid aggregates in the predentin.

• Suramin a polyanionic drug inducing experimental lipidosis and lysosomal accumulation of in secretory and postsecretory ameloblasts. Large lysosome-like vacuoles were positively stained with malachite green-aldehyde. The same was observed in secretory odontoblasts. Large defects were occurring during enamel formation. In suramin-injected rats electron dense aggregates were enhanced in number in the predentin and dentin support the occurrence of lipidosis in this animal model. Collagen fibrillogenesis was disturbed (Gritli et al. 1993, 1994). (Figures 8,9).

• Chloroquine, another drug that induces a lipidosis-like disease was also investigated. Both suramin and chloroquine indicate disturbances in the lysosomal apparatus, together with some alterations of enamel and dentin, but the relationship between these drugs and mineralization defects are difficult to demonstrate actually.

Until recently, the origin of dentin ECM phospholipids was not clarified. To investigate this assumption, we intravenously injected (3H) choline into rats and studied by radioautography how the radiolabeled precursor was incorporated into the cells and released. To avoid loss and translocation, the rats were perfused with MGA, the incisors dissected out and cut into thin 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.
Lipids extracted prior to demineralization have a composition close to biological membranes, namely sphingomyelin (SM), phosphatidylcholine (PC) and phosphatidylethanolamine (PE). The acidic phospholipids extracted after demineralization, associated to the mineral phase, include phosphatidyl inositol (PI), PC, PE, phosphatidylserine (PS), phosphatidic acid (PA) and cardiolipin (CL). They are similar in quality in enamel and dentin but differ in amount and proportions. This suggests that acidic phospholipids may play a role in the calcification process (Figures 9-12).

Genetic approaches of lipids in dental tissues

In the Fabry mice generated by the gene-targeting technique, lysosomes containing myelin-like structures were seen in odontoblasts. Enamel porosities contain cell membrane remnants, and accumulation of lysosomes inside odontoblasts. It was concluded from this investigation that disorders of glycol sphingolipid degradation seem to play only a minor role in odontogenesis (Goldberg et al. 2005) (Figure 13).
Neutral sphingomyelinase 2 (nSMase2), encoded by the Smpd3 gene, may provide a link between MV phospholipids and the PHOSPHO1 substrate. The nSMase2 gene is recognised to break down the membrane lipid sphingomyelin. The ablation of nSMase2 enzymatic activity by the deletion induced of a major portion of Smpd3, is recognized in the fro/fro mouse (Aubin et al., 2005). The chemically induced autosomal mutation named fragilitas ossium (fro/fro) is not related to any of the collagen defects shown to be responsible of most forms of osteogenesis imperfecta (OI).

Longitudinal sections of the mandible evidence shorter incisors in the fro/fro, about half-length of the heterozygote. 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 +/+ 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. Because the mineralization is impaired, the specific staining for CS/DS glycosaminoglycans is firmly enhanced using a specific (2B6) antibody in the fro/fro (Figure 17).

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, Goldberg et al., 2008). 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 ± 4% compared to +/+.

Conclusions

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. Extracellular Smpd amplifies BMP-4 induced osteocalcin synthesis (Kozawa et al., 2003). These results suggest that extracellular sphingomyelinase enhances the BMP-stimulated osteocalcin synthesis via ceramide in osteoblasts. During cartilage matrix vesicle-induced mineralization, there is a progressive disappearance of sphingomyelin, presumably due to its enzymatic degradation by nSMase2.
 Altogether, adding to what was observed in the fro/fro mutation suggest that proper sphingomyelin metabolism is essential for mineralization. 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.

Neutral sphingomyelinase 2 (nSMase2), encoded by the Smpd3 gene, may provide a link between MV phospholipids and the PHOSPHO1 substrate phosphocholine. nSMase2, is recognised to break down the membrane lipid sphingomyelin to produce ceramide and phosphocholine. The ablation of nSMase2 enzymatic activity by the chemically induced deletion of a major portion of Smpd3, as well as in the fro/fro mouse (Aubin et al., 2005; Stoffel et al., 2005). Smpd3 encode a membrane-bound enzyme that cleaves sphingomyelin and generate several bioactive metabolites (Khavandgar et al., 2013). The recessive mutation on what is called the fro/fro mice showed delayed mantle dentin mineralization, and as well as in enamel formation. Smpd3 expression in odontoblasts is also required for tooth mineralization.

Bone deformities in mouse models lacking a functional Smpd3 gene underscore the importance of sphingolipid metabolism in skeletal tissues. Stoffel et al. 2005) characterized the skeletal phenotypes of the Smpd3–/– mice as a chondrodysplasia and speculated a systemic role for Smpd3 in the regulation of the skeletal development. The poor mineralization of the skeletal tissues in the fro/fro mice is seen without any alterations of the homeostasis of Ca2+, Pi, and PPi (Khavandgar et al. 2011, 2013). This suggests that the loss of Smpd3 function affects ECM mineralization. The possibility of additional mechanism(s) can not be ruled out. It is supported by the fact that both Smpd3- and PHOSPHO1-deficient mice show bone mineralization defects. These enzymes may work in concert to increase the Pi levels in order to facilitate the nucleation of minerals inside the MVs and affects also subsequent stages of mineralization (Murshed. 2021).

Mechanisms of Mineralization

Once the nucleation core complex is formed, the membrane-enclosed domain is no longer essential for MV calcification. Our findings indicate that the MV core contains two main components:

1) a smaller membrane-associated complex of Ca2+, Pi, phosphatidylserine, and the annexins that nucleates crystalline mineral formation, and

2) a larger pool of Ca2+ and Pi bound to the proteins. The proteins appear to bind large amounts of mineral ions, stabilize the nucleation complex, and aid its transformation to the first crystalline phase.

Once nucleated, the crystalline mineral phase appears to feed on protein-bound mineral ions until external ions enter through the ion channels. (Wu et al., 1980). Ca2+ -bridging mechanism and phospholipid head group are recognized in the membrane-binding protein annexin V (Swairjo et al. 1995).

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References


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