Fibromodulin-Deficient Mice Reveal Dual Functions for Fibromodulin in Regulating Dental Tissue and Alveolar Bone Formation

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Key Words

Fibromodulin • Dentinogenesis • Amelogenesis • Osteogenesis • Collagen fibrillation • Enamel • Alveolar bone • Mineralization

Abstract

The extracellular matrix of newborn, 7- and 21-day-old fibromodulin-deficient (Fmod KO) mice was compared with age-matched wild-type (WT) mice. Western blotting of proteins from 21-day-old WT mice revealed that the molecular weight of Fmod is smaller in dental tissues (approx. 40 kDa) compared to alveolar bone extracts (approx. 52 kDa). Dentin matrix protein1 (DMP1) was slightly increased in Fmod KO versus WT tooth extracts. After chondroitinase ABC digestion, dentin sialophosphoprotein (DSPP) appeared as 2 strong bands (approx. 150 and 70 kDa) in incisors from 21-day-old Fmod KO mice, whereas the smaller-sized species of DSPP was nearly absent in WT molars and no difference was detected between WT and KO mice in molars. Dentin mineralization was altered in newborn and 7-day-old KO mice, but seemed normal in 21-day-old KO mice. DMP1 and DSPP may be involved in compensatory mechanisms. The enamel had a twisted appearance and looked porous at day 21 in KO incisor, and the outer aprismatic layer was missing in the molar.

Alveolar bone formation was enhanced in Fmod KO mice at days 0 and 7, whereas no difference was detected at day 21. We conclude that Fmod may control dental tissue formation and early maturation, where it acts mostly as an inhibitor in alveolar bone accumulation, excerpting its effects only at early developing stages. These dual functions may be related to the different forms of Fmod found in bone versus teeth.

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Abbreviations used in this paper

BSP	bone sialoprotein
DMP1	dentin matrix protein-1
DSP	dentin sialoprotein
DSPP	dentin sialophosphoprotein
EDTA	ethylenediaminetetraacetic acid
Fmod KO	fibromodulin-deficient
GAG	glycosaminoglycan
SEM	scanning electron microscopy
SIBLING	small integrin-binding ligand, N-linked glycoprotein
SLRPs	small leucine-rich proteoglycans
TEM	transmission electron microscopy
TGF- β	transforming growth factor β
WT	wild-type

Introduction

Small leucine-rich proteoglycans (SLRPs) are abundant in the extracellular matrix of dentin and bone. Fibromodulin (Fmod) is a class II-type SLRP that has keratan sulfate glycosaminoglycan (GAG) chains attached to the core protein [Iozzo, 1999]. It has been implicated in collagen fibrillation [Scott, 1996] and is considered as a TGF- β modulator [Soo et al., 2000]. Our previous studies on Fmod-deficient (Fmod KO) newborn mice showed they had impaired collagen fibrillogenesis in the predentin, altered dentin mineralization and reduction in thickness of the forming enamel [Goldberg et al., 2006]. Interestingly, small integrin-binding ligand, N-linked glycoprotein (SIBLING) [Fisher and Fedarko, 2003] expression was increased in the pulp and dentin in the newborn Fmod KO mice, indicating that compensatory mechanisms involving the SIBLINGs could contribute to dampening the effects of the Fmod deficiency in mineralized tissues. However, we felt that many of the functions of this molecule were not totally elucidated. Therefore, the time course study presented here is a continuation of our previously published work, adding new biochemical data to structural studies revealing the role of fibromodulin deficiency in the structure of mineralized tissues.

Materials and Methods

Newborn (day 0, corresponding to the formation of the crown for the molars), 7-day-old (formation of the root) and 21-day-old (end of the erupting period, the formation of the root being completed) Fmod KO and wild-type (WT) mice were euthanized. The mandibles were dissected, fixed and processed for light as well as transmission and scanning electron microscopy. Immunohistochemical studies were carried out using antibodies kindly provided by Dr. Larry Fischer (NIDCR, NIH, Bethesda, Md., USA) [Fisher et al., 1995].

For Western blot analysis, molars, incisors, mandible and maxilla were dissected from 21-day-old WT and Fmod KO mice and the proteins were isolated as described previously [Fisher et al., 1983]. Briefly, tissues were crushed to a fine powder at -80°C and incubated in a solution of 5 M guanidine for 24 h followed by 5 M guanidine/0.5 M EDTA for 48 h. Samples were dialyzed, lyophilized in water and then weighed. Equal amounts of protein were loaded onto a 4–12% Bis-Tris gel, transferred as described previously and probed with antibody to Fmod [Goldberg et al., 2006] at a dilution of 1:2,000. As the SIBLINGs in some cases were shown to be proteoglycans, some samples were digested with 0.002 units of chondroitinase ABC to remove GAG chains prior to Western blot analysis.

Results

Western Blotting

Silver staining of gels containing extracts from molars and incisors from 21-day-old mice showed that equal proportions of protein were present in samples from normal and Fmod KO mice (fig. 1a). Interestingly, in WT Western blots, the size of Fmod was clearly smaller in both incisor and molar (approx. 40 kDa) compared to mandible or maxilla (approx. 52 kDa). These data show, for the first time, a potentially different fragmenting pattern between bones and teeth that appeared as the animals aged (21-day-old vs. newborn mice; fig. 1b). As expected, no band was detected in Fmod KO mice. The intensity of the staining was slightly increased for dentin matrix protein 1 (DMP1) in the KO versus WT mice both in the incisor and molar. Prior to chondroitinase ABC treatment, anti-dentin sialophosphoprotein (DSPP) intensely stained the extract of the KO mice in the incisor, but was only weakly different in the molar. In contrast, after chondroitinase ABC treatment, 2 strong DSPP bands (approx. 150 and 70 kDa) were found in incisor of Fmod KO mice that were nearly absent in the WT samples. The staining was similar in molars of WT and Fmod KO mice.

Enamel

In the incisor, the thickness of the forming enamel in teeth of newborn Fmod KO mice was half of that of WT mice. At day 21, the enamel rods displayed a twisted appearance where large defects or porosities were seen in the outer border (fig. 1c). In the molars, enamel architecture was apparently normal, except that the aprismatic outer layer was still not formed at that time (compared to WT mice where it was already formed). This suggested that as a consequence of the mutation, the formation of enamel was delayed during the tooth formation and not even achieved at day 21 after eruption.

Dentin

Dentin was hypomineralized between days 1 and 7 in teeth of Fmod KO mice. The diameter of the collagen fibrils in predentin was enlarged in the inner half (Fmod KO: 25.6 ± 1.8 nm; WT: 16.3 ± 1 nm; $t > 0.01$) and the outer half (Fmod KO: 58.05 ± 1.9 nm; WT: 36.1 ± 0.9 nm; $t > 0.001$).

Dentin mineralization was impaired in newborn and 7-day-old Fmod KO mice. However, structural defects were not detected at day 21 neither in the incisor and the molar.

Fig. 1. **a** Silver staining of Western blots of WT and Fmod KO mice. **b** Western blot using an anti-fibromodulin antibody. **c** Scanning electron microscopy image showing normal dentin and a twisted appearance of a porous enamel. **d, e** The von Kossa staining shows that more bone is formed in newborn (NB) Fmod KO mice (**d**) versus WT mice (**e**). A = Ameloblasts; O = odontoblasts; B = bone. Scale bars = 50 μ m.

Alveolar Bone

In newborn and 7-day-old Fmod KO mice, bone formation was enhanced compared with WT mice (fig. 1d). This was less obvious at day 21, taking into account that the mandibular bone thickness is not homogeneous, a phenomenon that made comparison more difficult.

Discussion

The present investigation adds important new information to our previous studies [Goldberg et al., 2006], highlighted as follows.

The Tissue Specificity of Fmod: Dentin versus Bone

Western blots revealed different molecular weights of Fmod in the alveolar bone versus dental tissues. Fmod was first identified as a 59-kDa glycoprotein in immature cartilage [Oldberg et al., 1989]. In mature cartilage, it is a smaller glycoprotein. The molecular weight reported varies according to the sampling site (articular or tracheal) [Neame and Kay, 2000]. The core protein consists of 357 amino acid residues (42 kDa). It is tempting to speculate that the different forms of Fmod in bones versus teeth could have different biological functions. An alternative possibility is that Fmod lacks polylactosamine-modified N-linked oligosaccharide, as is the case during aging for

human articular cartilage [Roughley et al., 1996]. Here we used age-matched mice, therefore, the difference we saw could not be attributed to the aging processes. It is also possible that partial degradation occurs in dental tissues and not in bone. This may be due to the presence of MMP3, which was identified in the predentin [Hall et al., 1999].

Previously Unsuspected Effects of Fmod Deficiency on Dental Enamel

Fmod has been identified at day 14.5 of gestation in dental tissues but only in the outer enamel epithelium [Wilda et al., 2000]. At later stages of development (day 0), Fmod is not present in ameloblasts or in enamel, but is detected in the stratum intermedium alone [Goldberg et al., 2006]. Here we show that Fmod deficiency induces a structural alteration of the forming enamel resulting from an impaired mineralization of the rods. Rod/interrods were normally formed but displayed a twisted appearance and the enamel appeared porous, especially in the outer border of the incisor. It is likely that these porosities result from an impaired reabsorption of the matrix during the final phase of secretion and early postsecretory events, which could be one of the targets of Fmod deficiency. The resulting twisted rods could therefore be due to the compression during eruption of a defective mineralized enamel. This mechanistic interpretation needs to be further explored. Along these lines, it appears that amelogenesis is inhibited in the molar before the formation of the outer aprismatic enamel. Again, this finding supports the concept that the same group of ameloblasts is a target of the Fmod deficiency.

SIBLING Compensatory Mechanisms

We have previously shown and confirm here that Fmod deficiency induces an increased diameter of the collagen fibrils in the predentin. As a consequence, dentin formation is delayed at days 0 and 7 in the Fmod KO mice, and mineralization is impaired. However, at day 21, the dentin seemed normal. We hypothesized that the time-dependent recovery could be due to the overproduction of some SIBLINGs. Indeed, in newborn Fmod KO mice the immunostaining for dentin sialoprotein (DSP), DMP1 and bone sialoprotein (BSP) was enhanced, mostly in the pulp of the incisors. In the molar, DMP1 and BSP staining were also enhanced, but not for DSP. Osteopontin did not vary between WT and Fmod KO teeth and, therefore, was not implicated. The Western blot analysis shown here confirms that at day 21, DMP1 is increased in the KO mice, more in the incisor than in

the molar. After chondroitinase ABC digestion of day 21 extracts, Western blot analysis showed increased staining for DSPP in the incisor, but not for the molar. Thus, the present data support our previous conclusions [Goldberg et al., 2006]. Biological differences appear between the ever-growing incisor and limited growth molars. This should be taken into consideration in the interpretation of the specific deficiency and the effects of compensatory mechanisms. Some SIBLINGs are recognized as mineralization promoters, whereas others are inhibitors. DMP1 and BSP as nucleation promoters may compensate the deficiency due to the lack of Fmod [Tartaix et al., 2004].

Dual Functions of Fmod in Bone and Dentin

Fmod deficiency excerpts negative effects on dentinogenesis, causing delayed formation of dentin and altered mineralization. We confirmed that Fmod regulates collagen fibrillation in predentin and this function can interfere with dentin mineralization. We have shown previously that Fmod is abundant in odontoblasts judged by immunohistochemistry. These observations suggest an intracellular role still to be elucidated using other approaches such as in vitro cultivation and subcellular fractionation.

In contrast, Fmod deficiency enhances the number of mineralizing trabeculae in the alveolar bone of mouse mandibles, suggesting that Fmod normally acts as a repressor of alveolar bone formation. This was obvious at days 0–7 but less at day 21. Again, compensatory mechanisms probably interfere with the initial effect. These preliminary results reveal previously unknown dual or diverging functions for Fmod that need to be further investigated.

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