Abstract: This study focused on the abnormality of dentin matrix to clarify the pathogenesis of human dentinogenesis imperfecta (DI). Dentin matrix of mandibular molars from wild type (Zip13+/+) and Slc39a13/Zip13 knockout (KO) mice were examined histologically. Hematoxylin and eosin, as well as silver staining, were used for light microscopy. Samples were also observed under backscattered and transmission electron microscopy. Immunohistochemistry using type I collagen was also carried out. Results showed that Zip13-KO mice exhibited 1) lamellated dentin matrix with accentuated incremental lines under the light microscope, 2) abnormal collagen fibers and round shape collagen molecules in transmission electron microscope, and 3) unusual immunoreaction to collagen type I. The similarity in the histological features of dentin in Zip13-KO mice to human DI indicates that Zip13-KO mice may be used as models for investigating the mechanism of human DI.

Key words: Dentinogenesis imperfecta, Collagen fiber, Lamellar structure, Slc39a13/Zip13

Introduction

Dentinogenesis imperfecta (DI) is broadly divided into two clinical types; dentin dysplasia accompanied by osteogenesis imperfecta (DI type I) and dysplasia that appears only in dentin (DI type II). DI type I, reported to have a higher incidence than DI type II, is due to mutation of collagen type causing defective quality and quantity of the protein1-2). Many morphological studies have shown the histological features of dentin matrix in DI type I. The abnormality in collagen fibers was reported to be the major cause for the presence of incremental lines and sudden structural changes characterized by lamellated calcification of dentin matrix3-8).

Normally, calcification occurs when there is deposition of inorganic matrix in collagen fibers secreted by odontoblasts. However, in DI, unusual lamellar calcification occur wherein minerals are deposited in between substrates and fibers, believed to be due to peculiar collagen fibers produced by the cells. So far, only few studies showed the detailed relationship between the cells and the aberrant collagen fibers causing persistent lamellated calcification of dentin matrix9). In this regard, clarifying the biological nature of odontoblast may demonstrate the pathogenesis of DI.

Recently, the unknown feature of zinc transporter gene in Slc39a13/Zip13 knockout mice (KO, Zip13-/-) was investigated in order to elucidate the role of zinc transporters in the body. Zip13 gene together with bone morphogenetic protein (BMP) is associated with the formation tissues like bone, teeth and blood vessels. Zip13 gene is also involved in the regulation of signal transduction of TGF-β in cell proliferation and differentiation, embryonic development, connective tissue formation and bone formation. Likewise, Zip3 gene present in mouse is critical in signal transduction of BMP/TGF-β in the generation of connective tissue collagen having similar role in humans. Moreover, Zip3-KO mice exhibited tooth anomalies, reduced bone mass and abnormal biosynthesis of collagen type I during development. Zip13 gene has also been identified to be responsible for Ehlers-Danlos syndrome in human10-15). At 4 weeks, mice lacking in Zip13 gene showed severe dysplasia of root dentin, narrowing of pulp cavity and root shortening, which are some of the clinical features of human DI type I. However, since significant attrition of enamel was not observed, the clinical findings do not completely correspond to the clinical features of human DI type I. Consequently, questions arise on the role of Zip13 gene in dentin. Since quantitative and qualitative defects in collagen type I comparable to those found in DI can be seen in Zip13-KO mice, examination of Zip13-KO mouse dentin may bring an insight in the pathogenesis of human DI.
The study focused on the histological analysis of dentin matrix to elucidate the pathogenesis of human DI and whether Zip13-KO mouse can be used as a model for the development of the disease.

Materials and Methods

The experiment was based on the provisions and guidelines of Experimental Animal Committee and Animal Breeding Facility of Tsurumi University, School of Dental Medicine.

Experimental materials

The mice used in this experiment were Zip13 wild type (WT) and Zip13-KO mice produced by Fukada et al14).

Experimental methodology

Zip13-KO mice (n=24) and WT mice (n=24) were divided at 2, 4, 6 and 12 weeks. The dentin matrix of mandibular first molars was the tissue sample subjected to light and electron microscopy as well as immunohistochemistry (IHC).

Light microscopy

Hematoxylin and eosin stain (HE stain)

The amount of dentin formation stained with HE stain was measured using image analysis software (image-pro plus version 6.2) from the root dentin on the pulp side to the outermost layer based on the crest of alveolar bone without including the cementum (n=8).

The experimental mice were euthanized by cervical dislocation and the mandible was removed from the skull. After removing excess tissues, the specimens were fixed in 10% neutral-buffered formalin solution and embedded in paraffin wax following routine histologic procedure. Sections were cut in a buccolingual direction and stained with HE stain.

Silver staining

Silver impregnation was carried out according to Gomori G16). Paraffin sections were oxidized in 0.5% potassium permanganate aqueous solution and bleached in 2% oxalic acid aqueous solution. Then after, the specimens were fixed in sodium thiosulfate, dehydrated and mounted with glycerin-based mounting solution.

Ultra-structural technique

Transmission electron microscopy (TEM)

Perfusion fixing was carried out by inserting the catheter from the left ventricle using 25 % glutaraldehyde (0.2M cacodylate buffer, ph 7.4) under diethyl ether anesthesia. After removal of the mandible, the specimens were decalcified in EDTA for 2 weeks, fixed for 2 hours in 1% osmium tetroxide solution, dehydrated in increasing series of alcohol and embedded in epoxy resin (EPOK812, Tokyo). Ultrathin sections were cut, subjected to uranium acetate-lead citrate double staining and observed under TEM (JA1200EX, JEOL, Tokyo).

Backscatter electron diffraction/image (BEI)

Non-decalcified samples for TEM were embedded in epoxy resin, sectioned buccolingually parallel to the long axis of the tooth passing through the pulp cavity using a diamond knife. Images were reflected in carbon deposition EMPA (JXA8900, JEOL, Tokyo).

Immunohistochemistry stain (IHC)

Specimens were fixed in 4% paraformaldehyde, decalcified with EDTA and embedded in paraffin wax. Sections were subjected to enzymatic treatment and blocking with DAKO Protein Block at room temperature. Primary antibody against collagen type I (Ab34710) with a concentration of 0.4 mg/ml was allowed to react at 4 oC. After washing, the secondary antibody, anti-rabbit Ig Biotin (DakoE0432) was allowed to react at room temperature. Then after, HRP-labeled streptavidin (Nichirei 426062) was allowed to react at room temperature. Antigenic sites were revealed using hydrogen peroxide and diaminobenzidine (DAB). Slides were then dehydrated, sealed with glycerin-based mounting solution and observed under the light microscope.

Results

Light microscopy

Dentin formation

In WT mice, the dentin from the pulp side to the outermost surface layer relative to the crest of the alveolar bone (not including the cementum) formed an average thickness of 155 μm at 12 weeks. Consequently, dentin formation in WT mice was 2.5 μm/day. On the other hand, dentin formation in Zip13-KO mice was almost

Figure 1. Schematic diagram of Zip13-KO mouse dentin formation.
a: 2 to 3 weeks, b: 4 to 6 weeks, c: 12 weeks
OD: odontoblast, PD: pre-dentin, D: dentin, PL: periodontal ligament, LS: lamellar structure
a: the average amount of dentin formed was 1.7 μm/day, b: observation after 30 days, c: first area to be intensely stained

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similar with an average of 1.7 μm/day.

From this point, in Zip13-KO, it took about 30 days for the dentin matrix to be homogenously stained with hematoxylin, which was not observed until the 16th day (Fig. 1).

**Morphology of dentin matrix**

In WT mice at 2 weeks, examination of specimens under HE stain showed unerupted tooth with several layers of clear predentin as well as odontoblasts (Fig. 2a). At 4 weeks, dentinal tubules and dentin formation adjacent to the pulp were very clear, which continued at 6 and 12 weeks until it has completed its formation.

In Zip13-KO mice at 2 weeks, no difference in dentin matrix was observed compared to WT mice. However, at 4 weeks, lamellated structures stained with HE stain were evident, resembling accentuated incremental lines in the matrix (Fig. 2f). At 6 weeks, the dentin layer near the pulp was further stained homogenously with HE stain although lamellated structures were still noted (Fig. 2g). Homogenous staining of dentin was achieved at 12 weeks (Fig. 1h). The predentin in Zip13-KO mice was generally thinner compared to WT mice and odontoblasts were more irregular in shape in Zip13-KO mice at each week compared to WT mice (Fig. 2e-h).

**Silver impregnation findings**

In WT mice, the dentin matrix fibers run parallel to one another in a regular manner deeply stained with silver (Fig. 3a, b). On the other hand, in Zip13-KO mice, disturbance in the orientation of dentin matrix fibers was remarkable (Fig. 3c, d). Furthermore, the dentin matrix fibers in WT mice were homogenously stained while some of the matrix fibers in Zip13-KO mice were lightly stained (Fig. 3c, d). The difference in staining intensity was also observed in HE stain.

**Ultra-structural observation**
In WT mice, the dentinal tubules were relatively thin but became prominent towards the pulp (Fig. 4a). On the other hand, in Zip13-KO mice, the dentinal tubules away from the pulp were lightly stained (similar in HE stain) compared to those on the pulp side with a 256-level gray scale showing high signal reflection of electrons (Fig. 4b).

Furthermore, BEI of Zip13-KO mice showed numerous relatively thick dentinal tubules running in the direction of the pulp coming from the other half of the dentin root surface (Fig. 4b).

**TEM findings**

In WT mice, a periodic characteristic of collagen type I collagen fibers measuring 64 nm, indicates a substantial uniform thickness of collagen fibers arranged in a certain direction (Fig. 5a). On the other hand, in Zip13-KO mice, the collagen fibers showed sequences of small, clear, periodic structure with granular appearance and large numbers of fibers were arranged sparsely in various directions forming alternating layers (Fig. 5b, c). Those granular structures observed formed continuous linear structures (Fig. 5d). Initially, the linear structures were thought to be cross-sections of the matrix fibers although most images observed were oblique sections of the short fibers. Hence, the granular structure observed in TEM was not considered cross-sections of the collagen fibers.

**IHC findings**

In WT mice, a weak intensity of collagen type I across the root dentin was generally observed compared to the intense staining of the adjacent cementum (Fig. 6a-d). On the other hand, in Zip13-KO mice, cementum reacted to collagen type I as well. Although the intensity was weak, the matrix showed relatively strong immunoreaction from 2 to 12 weeks (Fig. 6e-h). In particular, the depth of the root surface from 26.9 μm to 50.3 μm showed consistent lamellated appearance similar to those observed in HE stain (Fig. 6g, h). However, IHC reaction was weak in the pulp side (Fig. 6g, h).

**Discussion**

In general, histological examination of dentin in people affected with DI is characterized by deficient calcification, lamellated dentin and abrupt structural changes. Several years ago, Ivancic distinguished the odontoblasts responsible for laying down circumpulpal dentin from those that formed the mantle dentin17). It was mentioned that the cells that formed the mantle dentin were destroyed and that circumpulpal dentin was formed by another odontoblast. Moreover, Harold et al showed through IHC, that there is a resemblance between the dentin in DI and the mantle dentin18). In this study, dentin matrix of Zip13-KO mice had disordered dentinal tubules and the partially amorphous structure of dentin was similar to those seen in human DI. Moreover, deficient calcification of dentin with lamellated structure and irregular patterns resemble those found in human DI.

In this study, lamellated and dysplastic dentin appeared on day 16, which was about 2 to 3 weeks. This observation coincided with the study of Fukada et al.15) in which the onset of the function of Zip13 gene was mentioned to be 2 to 3 weeks. Furthermore, the demarcation line formed between the dentin near the pulp and the lamellated structure occurred on day 30, which is roughly 4 to 5 weeks. The cause of the irregular and lamellated structure is not clearly known and considerable investigation is necessary. Nevertheless, limited studies related to odontoblastic intracellular signaling have been inferred. Melatonin and estrogen are known to be associated to the biologic rhythm of the cell19-21). In this study, since zinc transporter in the zinc-finger is missing in Zip13-KO mice, the incremental lines from the circumpulpal dentin and

![Figure 3. Zip13-KO root dentin matrix fibers](image)

![Figure 4. 12 weeks, root dentin](image)
lamellated structure may be brought about by the disturbance in the biological rhythm of odontoblasts.

The lamellated structures observed in light microscopy corresponded to those seen in TEM characterized by thin and scarce collagen fibers in various sizes and orientations scattered within the granular matrix.

A study in Zip13-KO mice showed abnormal modification of collagen in golgi apparatus of collagen-secreting cells\(^{14,15}\). The loss of zinc transporter in Zip13 explained the distribution of tropocollagen molecules within the cytoplasm. In the present study, although detailed explanation about the granular structure and variously sized fine fibers is unknown, it is possible that production of non-collagenous proteins, unusual modification of collagen and secretion of polysaccharides occurred. Essentially, the cross-linked structure of collagen molecule took place in the golgi apparatus. However, the change in collagen fibers was caused by EDTA during demineralization resulting to the granular appearance.

Thin collagen fibers coincided with the lamellated structures observed in the calcified layer in BEI. It was also observed that hydroxyapatite crystals were deposited among the dense collagen fibers.

The qualitative and quantitative features of collagen fibers in Zip13-KO mice were examined by IHC. A strong immunoreaction specifically at the site of lamellated structure was observed in Zip13-KO mouse dentin. On the other hand, immunoreaction to collagen type I of the same area was weak in WT mice. It is possible that odontoblasts in WT mice just like osteoblasts, synthesized different types of collagen not only type I but also type II or V and it is also possible that other extracellular molecules like elastin, proteoglycans and other glycoproteins were present in the connective tissue. Since the reason could not be elucidated, further consideration is necessary. Meanwhile, it is likely that the antibody reacted to other exposed epitopes that might be present in the abnormal structure of the collagen molecule. The molecular structure is necessary to be considered in future studies.

In this study, the abnormal secretion of collagen observed in dysplastic dentin was associated with the lack of Zip13 gene but the lamellated and calcified structure of dentin was associated with secretion of collagen substrates by odontoblasts. Moreover, the modification in the golgi apparatus resulted to unusual quality and quantity of collagen fibers. There are over 100 species of zinc-finger in the cell and Zip13 is only one of the Zip-family of genes. Nevertheless, it is suggested that Zip13 is candidate gene.
that may contribute to the development of human DI.

From the above results, Slc39a13/Zip13-KO mice used in this study presented morphological abnormalities in dentin very similar to those seen in human DI suggesting that it can be a useful model for the pathogenesis of human DI.

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References


