Morphometric analysis of cornea in the Slc39a13/Zip13-knockout mice

Takuya HIROSE1), Ippei SUZUKI1), Naoki TAKAHASHI1), Toshiyuki FUKADA2), Prasarn TANGKAWATTANA1,3)* and Kazushige TAKEHANA1)

1)Laboratory of Microanatomy, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan
2)Molecular and Cellular Physiology, Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Tokushima 770-8055, Japan
3)Department of Veterinary Anatomy, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

NOTE

Ehlers-Danlos syndrome (EDS) is a group of hereditary diseases caused by mutation of extracellular matrix (ECM)-related genes, such as COL1A1, COL3A1 and COL5A1 [6]. Major phenotypes of this syndrome include hyperextension of the skin, hypermobility of joints and fragility of blood vessels [4, 6]. Thus, EDS has been categorized into thirteen subtypes according to its different causes and symptoms [13]. SLC39A13/ZIP13 is a member of the SLC39/ZIP zinc transporter family localizing mainly in the Golgi apparatus of osteoblasts, chondrocytes, odontoblasts and fibroblasts [5]. This protein involves in transporting zinc from the Golgi apparatus to the cytoplasm and controls the nuclear shift of SMAD proteins in TGF-β1 and bone morphogenetic protein (BMP) signaling pathways [2, 3, 7, 16]. After the finding of mutation in SLC39A13/ZIP13 (ZIP13) of three gene types—i.e., Zip13, B3GALT6 and ZIP13, spondylodysplastic EDS-ZIP13 (spEDS-ZIP13: OMIM 612350) was recognized as a new EDS type. Its clinical expressions were classified into major criteria (including short stature, muscle hypotonia, and bowing of limbs) and minor criteria (including skin hyperextensibility, pes planus, delayed motor development, osteopenia, and delayed cognitive development). Besides, protuberant eyes with bluish sclerae were also included in the minor criteria [13]. spEDS-ZIP13 as a cause of thin cornea was also reported in 5-week-old Zip13-KO mice [7]. The cornea generally consists of five layers—i.e., anterior epithelium, anterior limiting membrane (Bowman’s membrane, usually absent in non-primates), proper substance (substantia propria, corneal stroma), posterior limiting membrane (Descemet’s membrane), and posterior endothelium. The proper substance with abundant collagen fibers and keratocytes occupies about 90% of the corneal thickness [1]. These collagen fibers configure into lamellae and each consecutive lamella usually arranged at almost right angle to each other [11]. Since EDS directly affects integrity of the ECM and eye problems were found in many cases of EDS subtypes and spEDS-ZIP13 [13], the ECM-rich cornea should be an affected organ of this syndrome. Therefore, this current study performed morphometric analysis of cornea in the wild type (WT) and spEDS-ZIP13 mice model.

The animal experiments were approved by the Committee on the Ethics of Animal Experiments of Rakuno Gakuen University (#VH15A6, June 15, 2015). Six C57BL/6; 129-SLc39A13tm1thir mice (RRBCR06217), being three Zip13-KO and three WT mice, were provided by RIKEN BRC via the National Bio-Resource Project of the MEXT, Japan. Mice of both groups were weaned at the age of 4 weeks. The 12-week-old mice were anesthetized by an intraperitoneal administration of 65 mg/kg pentobarbital (Somnopentyl®, Kyoritsu Pharmaceutical, Tokyo, Japan) and immediately euthanized by exsanguination. Then, the whole eyeball from each mouse was collected. The right fresh eyeball was embedded in OTC compound (Sakura Finetek Japan Co., Ltd., Tokyo,

*Correspondence to: Tangkawattana, P.: prasarn@kku.ac.th
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The decrease of collagen fibril diameter about 20% can decrease CFI about 36%. Reversely, the decrease of CFI of collagenous layer, CFI, and orientation of collagen fibrils. CFI value is affected by collagen fibril diameter and distance between collagenous layers, and CFI. Welch’s t-test was applied to compare mean ± standard errors of thickness of corneal epithelium and stroma, thickness and number of collagenous layers, and CFI. Students’ t-test was applied for diameter of collagen fibrils. Significant difference was accepted at P<0.05.

Table 1. Morphometric analysis of collagen fibrils in cornea of the WT and Zip13-KO mice group

| Analysis (mean ± standard error) | WT group | Zip13-KO group |
|----------------------------------|----------|---------------|
| Thickness of proper substances (µm) | 89.1 ± 1.0 | 41.1 ± 2.77a |
| Number of collagenous layers | 69.3 ± 0.9 | 68.7 ± 1.2 |
| Thickness of collagenous layers (µm) | 1.3 ± 0.01 | 0.6 ± 0.014a |
| Diameter of collagen fibrils (nm) | 27.4 ± 0.2 | 22.2 ± 0.99a |
| Collagen fibril index (%) | 34.0 ± 2.5 | 19.7 ± 2.08a |

a) Significantly different in mean ± standard error between groups at P<0.05.

Fig. 1. Light microscopic observation of the central cornea of the WT (a) and Zip13-KO mice group (b) with H&E staining. Thickness and appearance of the anterior epithelium (Ae) of both groups had no difference. Nevertheless, proper substance (Ps) in the Zip13-KO group was apparently thinner than that of the WT group. [Bars=10 µm].

Japan). 10-µm-thick sections being cut by cryostat (CM3050S, Leica) were stained with H&E. Three sections of each sample were used in investigating proper substance at the center of cornea and keratocytes in the center and limbus of cornea. The left eyeball was pre-fixed in 3.0% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 hr at room temperature and immediately post-fixed in 1.0% osmium tetroxide in 0.1 M phosphate buffer for 1 hr at room temperature. After washed with distilled water, the samples were dehydrated in a graded ethanol series and embedded in Quetol 812 (Nissin EM, Tokyo, Japan). The 60-nm-thick-sections were cut with a Reichert Supernova system (Leica, Vienna, Austria) equipped with a diamond knife, and mounted on a copper grid. Consecutive staining with 0.2% tannic acid + 10% ethanol in water for 15 min, 1.0% uranyl acetate for 5 min, and 1.0% lead citrate for 10 sec was performed. The samples were investigated with a transmission electron microscopy (TEM) (JEM-1200; JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV. Regards, diameters of 500 collagen fibrils randomly selected from each sample were measured. Collagen Fibril Index (CFI, ratio between collagen’s square surface and the sum of collagen and non-collagen’s square surfaces) in the ECM was analyzed by Image J software (version 1.48v; National Institutes of Health, Bethesda, MD, U.S.A.). Additionally, the number and thickness of collagenous layer were measured at 5 areas in each sample. Students’ t-test was applied to compare mean ± standard errors of thickness of corneal epithelium and stroma, thickness and number of collagenous layers, and CFI. Welch’s t-test was applied for diameter of collagen fibrils. Significant difference was accepted at P<0.05.

Apparent, there was no difference in thickness and histological appearance of the anterior epithelium between both groups. Nevertheless, thickness of the proper substance of the WT group (89.1 ± 9.1 µm) was approximately twice to that of the Zip13-KO group (41.1 ± 3.0 µm) with significant difference at P=0.018 (Fig. 1, Table 1). Although number of collagenous layers between the two groups (69.3 ± 0.9 layers in the WT group and 68.7 ± 1.2 layers in the Zip13-KO group) was not different (P=0.67), thickness of collagenous layer (1.3 ± 0.01 µm in the WT group and 0.6 ± 0.01 µm in the Zip13-KO group) was significantly different at P<0.001 (Fig. 2, Table 1). The diameter of collagen fibrils between the WT group (27.4 ± 0.2 nm) and the Zip13-KO group (22.2 ± 0.9 nm) was significantly different at P=0.030 (Fig. 2, Table 1). In addition, CFI of the Zip13-KO group (19.7 ± 2.0%) was significantly decreased upon comparing with that of the WT group (34.0 ± 2.5%) at P=0.011 (Fig. 2, Table 1). Each collagen fibril in the collagenous layer of the WT mice arranged parallel to each other while that of the Zip13-KO mice was in a random fashion (Fig. 2).

Morphology of keratocytes in both groups was found to have positional predilection (Fig. 3). Keratocytes in the central cornea of the WT mice and limbus of both groups appeared to have both elongated and bulb shape. But major keratocytic population in the central cornea of the Zip13-KO mice had elongated shape. Electron density in nucleus of the elongated keratocytes was lower than that of the bullet-shaped keratocytes (Fig. 4).

Although ocular symptoms and thin cornea in the spEDS patients and Zip13-KO mice were reported in previous studies [7, 14], subcorneal pathology pertaining to those cases was still unclear. Nevertheless, this study could reveal major vulnerable characteristics in the proper substance layer of cornea in the Zip13-KO mice, including thickness, keratocytic morphology, number of collagenous layer, CFI, and orientation of collagen fibrils. CFI value is affected by collagen fibril diameter and distance between collagen fibrils. The decrease of collagen fibril diameter about 20% can decrease CFI about 36%. Reversely, the decrease of CFI about 42% in this investigation would be the result of the spreading of collagen fibril distance. Such spreading would then be
fundamental on thickening of the collagenous layer. Thus, it is convincingly that the decrease of CFI is coherent with the widening distance between collagen fibrils. Reduction of ECM synthesis might be an initiation of these alterations via the blockade of Smad signaling pathway. In Zip13-KO fibroblast, Smad-signaling pathway having TGF-β1 stimulation was importantly for ECM synthesis [7, 8, 10]. The blockage of Smad-signalings inhibits Col1a2 mRNA expression resulting in the decrease of type I collagen synthesis in Zip13-KO fibroblast [7]. Although ZIP13 also existed in osteoblasts, chondrocytes, pulpal cells, and fibroblasts [7, 8], it was unclear whether keratocyte contained ZIP13. Since fibroblast and keratocyte derive from mesenchymal cell and ZIP13 gene is relatively highly expressed in the eye of murine species [7], ZIP13 should also exist in the normal keratocyte. Regards, decrease of collagen synthesis in keratocyte in the Zip13-KO mice should have mechanism similarly to that in the Zip13-KO fibroblasts.

Moreover, the differences in cellular morphology and electron density of nucleus of keratocyte in various localities should reflect the level of its transcription activity. Nucleus with a higher transcription activity must unfasten their chromatin granules so that transcription bubbles appear. Transcription could enrich RNA microenvironment to form euchromatin. Thus, the finely interspersing figures of the transcribed chromatin domains and RNA enriched microenvironments were maintained by the RNA accumulation in nucleus [9]. Such appearance finally destines the nucleus to have low electron density. In developing cornea, the bullet-shaped keratocytic stem cells with high transcription activity originally localizing in the limbus of cornea are predestined to move to the center of cornea [7, 15]. Such movement; therefore, could explain the existence of the bullet-shaped keratocytes both at the limbus and center of the cornea in the WT group. The bullet-shaped keratocytic stem cells at the limbus of cornea in Zip13-KO mice also travelled toward the center of cornea. During the relocation, cellular elongation also occurred together with the diminish of its transcription activity [1, 11]. Hence, transcription activity of the bullet-shaped keratocyte with low electron dense nucleus must be higher than that of the elongated keratocyte with high electron dense nucleus. Since elongated keratocytes with high electron dense nuclei were the predominant population of the central cornea of the Zip13-KO mice, this area surely had low transcription activity for collagen synthesis. Such reduction then became a negative impact toward the integrity of proper substance of the cornea.

The assembly of collagen fibrils to establish certain fibril diameter in cornea and elsewhere is chiefly regulated by various proteoglycans (PG) and glycosaminoglycans (GAG) [12, 18]. It has been known that dermatan sulfate increases collagen fibril diameter, but keratan sulfate inhibits the increase of collagen fibril diameter in the cornea [17]. In the Zip13-KO mice, the imbalance of these substances would certainly affect the fibril diameter. It is the fact that PG consists of core protein and GAG chains. Since the major PG in cornea is lumican and the GAG chain of lumican is keratan sulfate [5, 18], the loss of balance of PG components—e.g., increase of lumican or decrease of other PG (such as decorin which has dermatan sulfate as GAG chain) [18],

Fig. 2. TEM observation of collagen fibrils in the collagenous layer (CL) of the central cornea of the WT (a, b) and Zip13-KO mice group (c, d). Thickness of CL, collagen fibril diameter and CFI in the WT group was higher than those in the Zip13-KO group. While almost all collagen fibrils in the WT group had cross-sectional alignment (b, arrowhead), those in the Zip13-KO group aligned in both cross-sectional (d, arrowheads) and longitudinal pattern (d, arrows). [Bars=100 nm (b, d) and 200 nm (a, c)].
would certainly affect the abnormal fibril assembly and decrease diameter of collagen fibrils. At present, the relationship between ZIP13 and GAG synthesis is still unclear. Such abnormality would decrease CFI and alter thickness and orientation of collagen fibrils. Because the transparency of cornea is regulated by a regular arranging pattern of collagen fibrils, the high degree of fibrillar misalignment would be the cause of opacity of cornea [5]. However, opaque cornea was not observed in the Zip13-KO mice group. It is possible that corneal opacity occurs only in the progressive spEDS cases [13].

This morphologic analysis could reveal the vulnerable characteristics of corneal stroma in the Zip13-KO mice. The understanding in pathologic mechanisms of various abnormalities would contribute necessary information for future research towards the alleviation of spEDS.

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