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SHORT COMMUNICATION

Morphometric analysis of thoracic aorta in *Slc39a13*/*Zip13*-KO mice

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Abstract

Ehlers–Danlos syndrome (EDS) is a collection of inheritable diseases involving the musculoskeletal, integumentary and visual systems. Spondylodysplastic EDS-*ZIP13* (spEDS-*ZIP13*: OMIM 612350) was recently defined as a new form of EDS. Although vasculitis has been found in many spEDS-*ZIP13* patients, vascular pathology has not been included as a pathognomonic lesion of this type of EDS. We investigate the morphometry of the thoracic aorta in wild-type and *Zip13*-knockout (*Zip13*-KO) mice. Our assessment found abnormalities in the number and morphology of elastic and cellular components in the aortic wall, especially the tunica media, of *Zip13*-KO mice, indicating aortic fragility. Accordingly, our major findings (vascular smooth muscle cells with small nuclei, small percentage of elastic membrane area per tunica media, many large elastic flaps) should be considered vulnerable characteristics indicating fragility of the aorta in patients with spEDS-*ZIP13*.

Keywords Aorta · Elasticity · Elastic membrane · Morphometry · *Slc39a13*/*Zip13*-KO mouse

Introduction

Ehlers–Danlos syndrome (EDS) is a collection of hereditary diseases that manifest as skin hyperextension, joint hypermobility and ocular lesions (Deren-Wagemann et al. 2010). These manifestations are accompanied by an abnormal extracellular matrix (ECM) caused by mutations of many ECM-related genes (Malfait et al. 2017). Spondylodysplastic EDS (spEDS), or spondylocheirodysplastic EDS (SCD-EDS) subtype, is known to involve three genes—*B4GALT7*, *B3GALT6* and *SLC39A13/ZIP13* (*ZIP13*) (Malfait et al. 2017). The mutation of *ZIP13* then led to spEDS-*ZIP13* (OMIM 612350) (Fukada et al. 2008). ZIP13, a member of the SLC39/ZIP family, is an intercellular zinc transporter located in the Golgi complex of osteoblasts, chondrocytes, pulpal cells and fibroblasts (Fukada et al. 2008). These cells are responsible for synthesis of ECM, including elastic fibers and the Smad signaling pathway (SSP) (Fukada et al. 2008; Kucich et al. 2002). Thus, if there is any blocking event in the SSP, ECM and elastin synthesis is likely disrupted. Because SSP blockade was reported in *Zip13*-KO fibroblasts (Fukada et al. 2008), we hypothesize that a similar blockade should be expected in the vascular system of *Zip13*-KO animals.

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The elastic membrane, composed of abundant elastic fibers, comprises the tunica media of arterial walls and is mainly responsible for their elasticity. Any abnormal events interrupting ECM and elastic fiber synthesis could have a negative impact on the integrity of this membrane and subsequently lessen arterial elasticity. Because elastic fibers are a major component of all vascular structures, this affliction is found not only in arteries but also in veins. This problem may manifest as arterial lesions in all of the spEDS subtypes, with complaints of severe varicosity of the legs and feet by spEDS-*ZIP13* patients (Dusanic et al. 2018; Fukada et al. 2008; Malfait et al. 2017).

Because the thoracic aorta is the major point of arterial rupture and the changes in ECM and elastin synthesis caused by reduced SSP activity is thought to be involved with the vascular pathology in *Zip13*-KO mice (Fukada et al. 2008), this study aims to reveal the characteristics that render a subject vulnerable to thoracic aortic injury. We conducted this study in *Zip13*-KO mice versus wild-type (WT) mice. Based on the detailed information concerning angiopathic events that we reveal in this study, we believe that it is essential for EDS scientists to consider vascular lesions as an spEDS criterion.

Materials and methods

Animals

This experiment was approved by the Committee on the Ethics of Animal Experiments of Rakuno Gakuen University (#VH15A6, June 15, 2015). Three WT mice and three *Zip13*-KO C57BL/6 mice (129-*Slc39a13*^{tm1thir} mouse strain: RBRCR06217) were supported by RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. All mice were weaned at 4 weeks of age. At age 12 weeks, they were anesthetized using intraperitoneal administration of pentobarbital 65 mg/kg (Somnopentyl®; Kyoritsu Pharmaceutical, Tokyo, Japan) and then immediately euthanized by exsanguination. The aorta was collected from each mouse and divided into three pieces to be used in light microscopic, immunohistochemical and transmission electron microscopic investigations, consecutively. The collected morphometric data gained from each investigation were then compared.

Light microscopy

The pieces of aorta from both groups were fixed in Bouin's fixative for 24 h at room temperature, then embedded in paraffin. Three 4-μm-thick sections from each aorta were cut and separately stained with hematoxylin-eosin (H&E), elastica van Gieson (EVG), or Sirius red (SR).

Immunohistochemistry for α-smooth muscle actin

The pieces of the aorta from both groups were fixed in 4.0% paraformaldehyde–phosphate buffer solution for 24 h at room temperature. The samples were cut into 4-μm-thick sections. We used Histofine® MOUSESTAIN KIT (Nichirei Bioscience Inc., Japan, Tokyo) according to the instruction manual and smooth muscle anti-α-actin antibody (α-SMA, ab5694; Abcam, Japan, Tokyo) as the first antibody.

Transmission electron microscopy

The other pieces of aorta from both groups were prefixed in 3.0% glutaraldehyde. The samples were observed by TEM (JEM-1220; JEOL, Tokyo, Japan) according to conventional methods (Hirose et al. 2015).

Results

Light microscopy

The H&E-stained sections showed that there was no abnormality in the tunica interna or tunica intima in either mouse group (Fig. 1a, b). In the EVG-stained sections (Fig. 1c, d), wavy elastic membrane in the tunica media of the WT and *Zip13*-KO mice was clearly observed, whereas there was no difference in the thickness of the tunica media or the number of elastic membranes in the WT versus *Zip13*-KO mice. Nevertheless, the percentage of the elastic membrane area per tunica media in the *Zip13*-KO mice was lower than that in the WT mice (Table 1). The SR-stained sections showed a collagenous patch underneath each elastic membrane and a thick collagenous band in the tunica externa (Fig. 1e, f). The percentages of collagen fiber area per tunica media were 21.3 ± 0.2% in WT mice and 21.4 ± 0.4% in *Zip13*-KO mice, with the difference not significant (Table 1).

Immunohistochemistry for α-smooth muscle actin

The positive immunohistochemistry for α-SMA confirmed that all the cells interspersed between the elastic membrane of the tunica media in the aortas of both mouse groups were vascular smooth muscle cells (VSMCs) (Fig. 1g, h). The H&E-stained sections, however, showed no significant difference in the number of VSMCs between the WT group and the *Zip13*-KO group. In contrast, the nucleus per VSMC was smaller in the *Zip13*-KO mice than in the WT mice (Table 1).

Transmission electron microscopy

The transmission electron microscopy evaluation clearly showed wavy elastic membrane and VSMCs in the tunica

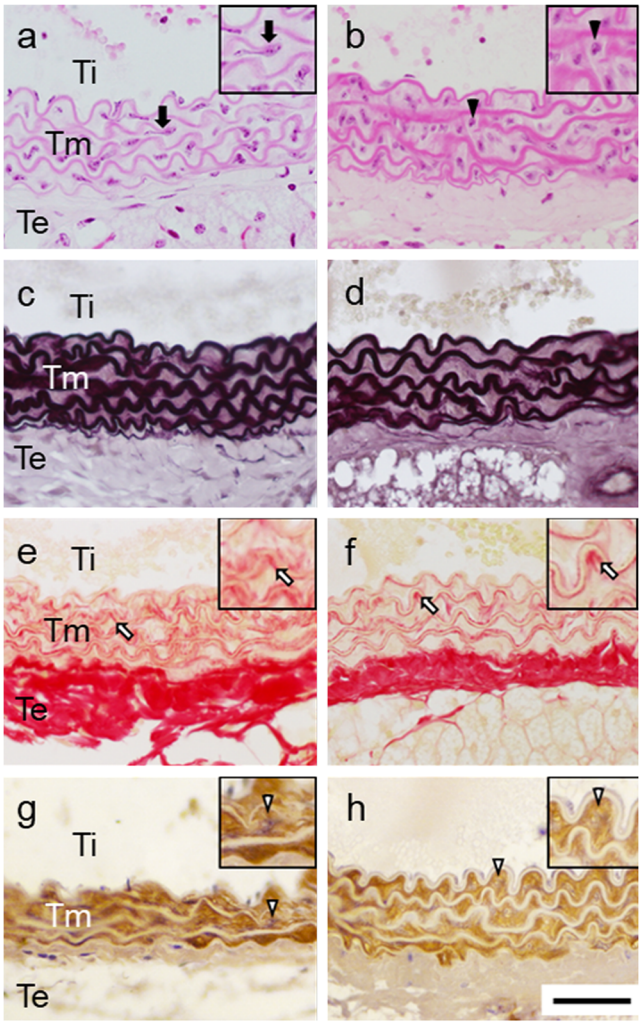

Fig. 1 Light microscopic observation of the thoracic aorta in wild-type (WT) mice (**a**, **c**, **e**, **g**) and *Zip13*-KO mice (**b**, **d**, **f**, **h**) after applying hematoxylin-eosin (H&E) (**a**, **b**), elastica van Giesen (EVG) (**c**, **d**) and Serius red (**e**, **f**) stains and smooth muscle anti-α-actin SMA (α-SMA) (**g**, **h**). Generally, the tunica intima (Ti) of the aorta in both mouse groups was still intact. Cells in the tunica media (Tm) were oval in WT mice (**a**, black arrow, inset) and round in *Zip13*-KO mice (**b**, black arrowhead, inset). Wavy elastic membrane in the tunica media is clearly shown, especially with EVG staining. The percentage of elastic membrane area per tunica media was smaller in *Zip13*-KO mice (**d**) than in WT mice (**c**). A small patch of collagen fibers attached under the wavy elastic membrane of tunica media (**e**, **f**, white arrow, inset) and a thick band of collagen fibers in the tunica externa (Te) are clearly visible in the aorta after Sirius red staining (**e**, **f**). Vascular smooth muscle cells between the elastic membrane of the tunica media stained positive with α-SMA (**g**, **h**, white arrowhead, inset). In *Zip13*-KO mice, the differences with WT mice were VSMCs with small nuclei and a small percentage of elastic membrane area per tunica media. Bar = 50 μm

media (Fig. 2). In addition, the elastic membrane seemed to be thinner in the *Zip13*-KO mice than in the WT mice. However, a significant difference in the average thickness of the elastic membrane between the two groups was not seen. Both small (≤ 4 μm in length) and large (> 4 μm in length) elastic flaps were seen (Fig. 2d, arrowhead) emanating from the elastic membranes of the two groups. It was apparent that there were more small flaps but fewer large flaps in the WT mice than in the *Zip13*-KO mice. Note that the difference between the groups was statistically significant for the large flaps (Table 1).

Discussion

Although muscle, skeleton, integument and corneal abnormalities had already been reported in *Zip13*-KO mice (Fukada et al. 2008; Hirose et al. 2018; Hirose et al. 2015), the morphology and pathology of the thoracic aorta in these mice remained unclear. Our major findings concerning the thoracic aorta of *Zip13*-KO mice—VSMCs with small nuclei, small percentage of elastic membrane area per tunica media, many large elastic flaps—suggest a fragile, vulnerable thoracic aorta.

Elastin is secreted by VSMCs in blood vessels (Eble and Niland 2009). Immunohistochemistry analysis showed that the cells between the elastic membranes in the tunica media of both groups were VSMCs. The nucleus of each VSMC in the *Zip13*-KO group was smaller than that in the WT group. The nuclear size of VSMCs in the tunica media of the thoracic aorta in *Zip13*-KO mice concurs with its low transcription activity. Thus, mutation of *Zip13* would retard the SSP, subsequently decreasing elastin expression in VSMCs. Inhibition of the SSP in *Zip13*-KO fibroblasts was reported (Fukada et al. 2008). Additionally, stabilization of elastin mRNA responsible for the synthesis of elastin in the SSP is the function of TGF-β1 (Kahari et al. 1992; Kucich et al. 2002, 1997; Rosenbloom et al. 1993; Zhang et al.1995). It is known that TGF-β1 is important in remodeling vascular structures by stimulating growth of VSMCs and Smad proteins are expressed in VSMCs—suggesting that retardation of the SSP would subsequently decrease elastin expression in VSMCs of the *Zip13*-KO. Lowering elastin production could affect the morphometric quality of mural construction, especially the elastin and cellular population in aortic tunica media, leading to decreased tunica media thickness and aortic elasticity. The appearance of vascular lesions could be a sequela of high-pressure loading onto a less elastic (more rigid) wall.

Previous studies have reported severe varicosity in the lower extremities of spEDS patients without other cardiovascular symptoms (Dusanic et al. 2018; Fukada et al. 2008; Giunta et al. 2008). In the present study, we used 12-week-old *Zip13*-KO mice. These mice did not show aneurysms or arterial ruptures, while abnormalities in the tunica media of the thoracic aorta were observed.

Wagenseil et al. reported that loss of aortic elasticity in elastin-insufficient ($Eln^{+/-}$) mice was not caused by loss of the thin elastic membranes (Wagenseil et al. 2010). By contrast, $Fbn1^{+/mg\Delta}$ mice, a model of Marfan syndrome, have thin and disrupted elastic membranes similar to *Zip13*-KO mice

Table 1 Morphometric analyses of the thoracic aorta of WT and *Zip13*-KO mice

Analysis	WT	*Zip13-KO*
Thickness of tunica media (μm)	55.9 ± 1.3	54.0 ± 1.2
Thickness of elastic membrane (nm)	1877.6 ± 12.0	1821.8 ± 41.0
Number of elastic membranes	5.4 ± 0.2	5.1 ± 0.1
Percentage of elastic membrane area per tunica media	54.2 ± 1.0	49.6 ± 1.0[a]
Percentage of collagen fiber area per tunica	21.3 ± 0.2	21.4 ± 0.4
Number of vascular smooth muscle cells (cells/0.01 mm^2)	32.8 ± 0.1	34.7 ± 2.9
Area of nucleus per vascular smooth muscle cell (μm^2)	34.4 ± 1.5	25.0 ± 1.6[a]
Number of elastic flaps (/0.01 mm^2)		
Small elastic flaps (length ≤ 4 μm)	5.2 ± 0.5	5.9 ± 0.8
Large elastic flaps (length > 4 μm)	4.1 ± 0.4	12.6 ± 1.9[a]

Data are given as the mean ± standard error (SE) and *t* test was applied to compare mean ± SE

[a] Significant difference in mean ± SE between groups in the same row at $P < 0.05$

(Mariko et al. 2011). This pathology may increase arterial stiffness and cause abnormal aortic wall architecture, leading to aneurysms (Mariko et al. 2011). Patients with Marfan syndrome caused by an *FBN1* mutation show a range of cardiovascular diseases. In particular, thoracic aortic aneurysms are frequently observed and aneurysm dissection is the leading cause of death in adult patients (Rurali et al. 2018). Thus, the characteristics of the thoracic aorta in *Zip13*-KO mice are similar to those in $Fbn1^{+/mg\Delta}$ mice, suggesting a vulnerability to low elasticity, aneurysms and aortic rupture in *Zip13*-KO mice.

In conclusion, abnormalities in the mural architecture of the thoracic aorta may increase the risk of vascular aneurysm or aortic rupture in spEDS-*ZIP13* patients.

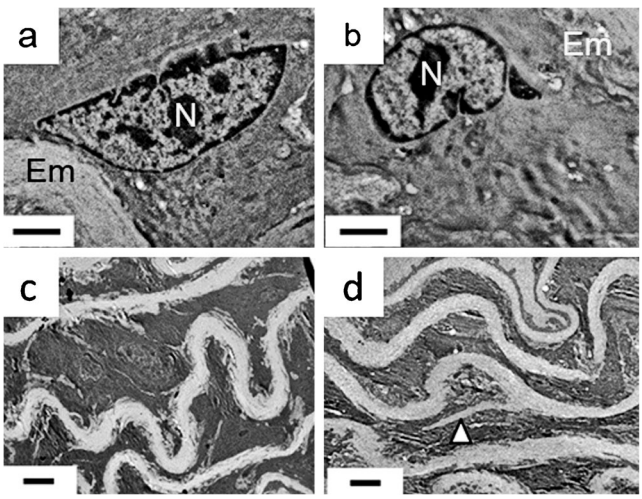

Fig. 2 Transmission electron microscopy of the elastic membrane in aortic tunica media of WT mice (**a**, **c**) and *Zip13*-KO mice (**b**, **d**). Nucleus (N) of the cell, seen within the wavy elastic membrane (Em) of *Zip13*-KO mice, was apparently smaller than that of WT mice. Elastic flaps emanating from the main trunk of the elastic membrane were usually larger in *Zip13*-KO mice (**d**, arrowhead) than in WT mice. *Zip13*-KO mice had many large elastic flaps compared with WT mice. Bars = 2.5 μm

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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