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ORIGINAL PAPER

Expression of Elastin, F-Box and WD-40 Domain-Containing Protein 2, Fibrillin-1, and Alpha-Smooth Muscle Actin in Utilized Blood Vessels for explant culture—A New 3D in Vitro Vascular Model from Bovine Legs

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Abstract

Elastic fibers of the internal and external elastic laminae maintain blood vessel shapes. Impairment of smooth muscle cell function leads to vascular disease development. F-box and WD-40 domain-containing protein 2 (FBXW2) is associated with elastic fibers and osteocalcin expression for bone regeneration in the periosteum. Here, it is hypothesized that FBXW2 has different roles in periosteum and blood vessels. Furthermore, if FBXW2 would be a component of elastic fiber of blood vessels, FBXW2 would be expressed where the well-known components elastin and fibrillin-1 are expressed. For this purpose, explant culture of blood vessels from bovine legs were performed for 5 weeks. It was found that elastin and FBXW2 were expressed within the elastic laminae, whereas fibrillin-1 was expressed around them. After explant culture, elastin and FBXW2 sustained the shape of the elastic fibers in the elastic lamina, whereas the fibrillin-1-rich layer became wide range and encompass toward intima and adventitia layers. Hematoxylin Eosin staining and immunohistochemistry of alpha-smooth muscle actin (α-SMA) revealed weakened media layer after 5 weeks culture. Although fibrillin-1 is a well-known component of elastic fibers and elastin, this study revealed that the location of fibrillin-1 is different from that of elastin, whereas FBXW2 is present in the same region as elastin from day 0 to week 5. In blood vessels, fibrillin-1 fibers around the elastic lamina may be oxytalan fibers. Thus, the proposed 3D in vitro model in this study is useful for identifying the mechanisms of vascular degradation.

Keywords Elastin · F-box and WD-40 domain-containing protein 2 · Fibrillin-1 · Alpha-smooth muscle actin · 3D in vitro vascular model

Highlights

- In arteries, fibrillin-1 is expressed strongly around elastic fibers.
- Explanted artery maintains its hollow shape after 5 weeks owing to elastic fibers.
- Fibrillin-1-rich regions narrowed blood vessel lumen after 5 weeks.
- Model will aid mechanistic study of vascular degradation-associated diseases.

Introduction

Atherosclerosis [1, 2] and aortic aneurysm [3] are life-threatening vascular diseases. The association between elastic matrix degradation and abdominal [4–8] or thoracic [9–11] aortic aneurysms is gaining attention. In the skin of patients with arterial tortuosity syndrome, discontinuous internal elastic lamina of small muscular arteries were observed [12]. The aging process of elastic fibers results in cardiovascular disease development [13] as well as affects the wrinkling and sagging of skin [14, 15] and causes reduced deglutition function [16]. When smooth muscle cell (SMC) proliferation is inhibited, the inflammatory effects increase [17]. A previous study examined SMCs in an in vitro study on cardiovascular diseases [18]. The

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correlation between elastic fiber and SMC was investigated [19, 20].

Elastic fiber is composed of elastin and fibrillin microfibrils [13, 21]. Mutated fibrillin-1 is related to Marfan syndrome [11, 22–24]. The relationship between fibrillin-1-derived asprosin and metabolic disorders gained attention [25]. Three different types of fibers (elastic, oxytalan, and elaunin fibers) are known to be present in the gingiva [26]; among these, oxytalan fibers are formed from microfibrils [16]. Microfibrillar scaffold proteins were identified and their role in elastic fiber association was investigated [27, 28].

In a previous study using bovine periosteum and blood vessels of bovine legs, Akiyama [29] reported that both elastin and F-box and WD-40 domain-containing protein 2 (FBXW2) are present in elastic fibers. Akiyama [30] investigated bovine periosteum and found that osteocalcin was located on FBXW2. Furthermore, FBXW2 and osteocalcin were separated from elastin under cell culture conditions without ascorbic acid [31]. However, in previous explant cultures of periosteum, osteocalcin was not expressed in the micro-vessels [29]. The aim of this study was to clarify whether (1) FBXW2 is a novel component of the elastic fibers of blood vessels, (2) fibrillin-1, another component of elastic fibers, is expressed in locations where elastin and FBXW2 are expressed, and (3) FBXW2 is separated from the elastic fibers in blood vessels under conditions encountered in the periosteum (without ascorbic acid). In this study, we highlight the relevance of presence of breakage in elastic fibers and change of positional relationship between elastin, FBXW2, and fibrillin-1, and proliferation or decrease of the intima, media, and adventitia.

Materials and Methods

Serial Sections

The use of bovine legs to obtain blood vessels was approved by the Osaka Dental University Regulations on Animal Care and Use (Approval No. 23-02007). In this study bovine legs were used within 24 h of the death of animals and no vital signs were present. In addition, this research did not involve any experiments on living animal or human participants.

Blood vessels (with a diameter of approximately 3 mm) were dissected into three parts from five randomly selected legs of five 30-month-old female or castrated male Japanese Black cattle (Kobe Chuo Chikusan, Kobe, Japan) as described previously [29, 32]. These cows grow in cattle farms all over Japan for meat product [32]. Figure S1 shows the region of dissected blood vessels (metacarpal artery and metatarsal artery). Blood vessels near two metacarpal bones and three tarsal bones were peeled to avoid contamination. Previously, ascorbic acid was found as essential for multilayered structure of periosteum-derived cells [31]. Approximately 30 mm long blood vessels from five cows were divided into three groups: (1) day 0; (2) 5-week culture in Medium 199 (12340-030; Gibco, Grand Island, NY, USA) with 5 mg/mL ascorbic acid (A0278-25G; Sigma, St. Louis, MO, USA); and (3) 5-week culture in Medium 199 (12340-030; Gibco) without ascorbic acid. Blood vessels of the three groups were obtained from the same cows.

For comparison of explant culture of periosteum, in CO_2 incubater, 15 ml medium 199 with 10% fetal bovine serum (FB1365/500; Biosera, Cholet, France) and antibiotics (penicillin–streptomycin solution for cell culture; Fujifilm, Tokyo, Japan) was changed once a week in 100 mm culture dish (Corning, NY, USA), as described previously [29]. On day 0 or after explant culture (5 weeks), the blood vessels were fixed in 4% paraformaldehyde. Serial sections were made from paraffin-embedded blocks. Paraffin blocks were sectioned for Hematoxylin Eosin (HE), Elastica van Gieson (EVG) stain, and immunohistochemistry.

Immunohistochemistry

Proteinase K (S3020; DakoCytomation, Glostrup, Denmark) was used as the antigen retrieval-agent for the antibodies against elastin, FBXW2, and fibrillin-1. Only for the alpha-smooth muscle actin (α-SMA) antibody, heat-induced epitope retrieval using Tris/ethylenediamine tetraacetic acid buffer (pH 9.0, 40 min; Diagnostic BioSystems, Pleasanton, CA, USA) was used. Antibodies against elastin (BA-4; sc-58756; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and FBXW2 (#PA518, 189; Invitrogen, Eugene, OR) were the same as those used in an earlier study [29]; however, in this study, the images shown are from cows different from those used in the previous study. To study elastic fiber components, antibodies against elastin, FBXW2, and fibrillin-1 (11C1.3; GeneTex, Inc., CA, USA) were applied. Antibody for elastin and antibodies for FBXW2 and fibrillin-1 were diluted 1:300 and 1:100, respectively. The antibody against α-SMA (1:500; ARG66381; Arigo Biolaboratories Corp., Hsinchu City, Taiwan) served as the marker for vascular smooth muscle cells. Three secondary antibodies, alkaline phosphatase-conjugated AffiniPure goat anti-rabbit IgG(H + L) (SA00002-2; Proteintech Group, Inc., IL, USA), alkaline phosphatase-conjugated AffiniPure goat anti-mouse IgG(H + L) (SA00002-1; Proteintech Group), and anti-goat IgG-AP (sc-2355; Santa Cruz Biotechnology, Inc., CA, USA) were used. For fluorescence immunohistochemistry, secondary antibodies, Alexa Fluor™ 488 goat anti-mouse IgG (H + L) #A327311:200 1 h (Invitrogen, Eugene, OR, USA) Mouse anti-goat IgG-CFL 594, #sc516243, 1:200 1 h (Santa Cruz Biotechnology,

Inc.), and 4',6-diamidino-2-phenylindole (Nacalai tesque, Kyoto, Japan) were used. For visualizing tissue samples, Perma Blue/AP (K058; Diagnostic BioSystems, Pleasanton, CA, USA) and PermaRed/AP (K049; Diagnostic BioSystems) was used and images were obtained using an all-in-one (bright field, phase contrast, fluorescence) microscope (BZ-X810; Keyence Japan, Osaka, Japan). X800 Viewer (Keyence) and X800 Analyzer software (Keyence) were used to analyze all images. Three negative controls (mouse, goat, and rabbit) for primary antibodies are tested. For mouse and goat negative controls, Proteinase K, only antibody diluent (S3022; DakoCytomation) without primary antibodies, and secondary antibodies were used. For rabbit negative control, heat-induced epitope retrieval, antibody diluent, and secondary antibody were used. For comparison with HE staining, BCIP-NBT Solusion (#19880-84, Naclai tesque) was used for fibrillin-1 immunostaining because of high sensibility. Staining intensity of elastin, FBXW2, and fibrillin-1 was analyzed using Image J 1.54 f (http://imagej.org, NIH). For analysis, straight lines were drawn from lumen to adventitia, and "Analyze" was chosen. "Plot Profile" and then Gray value from start of line to end of line was gained. Horizontal axis showed distance from start point. Quantitative analysis was performed using X800 Analyzer software (Keyence) to clarify the positive stained area without background staining. "Bright field" was selected as the image type. A target area was created and total of the extract area with recognized brightness was calculated. The number of counted areas and the total area were calculated with excel files and colored images (elastin, FBXW2, and fibrillin-1 was light purple, red, and green, respectively). The excel file represents law data for quantitative analysis. Statistical analyses were not performed.

Results

The expression of elastin, FBXW2, and fibrillin-1, analyzed using serial sections, is presented in Figs. 1, 2. Expression of elastin and FBXW2 (strong expression in internal and external elastic laminae) was different from that of fibrillin-1 (weak expression within laminae, high expression outside elastic lamina). Cross-sectional analysis of staining intensity also revealed that although background staining of FBXW2 was observed, elastin and FBXW2 were expressed in elastic laminae, in which the expression of fibrillin-1 was weaker than that of elastin and FBXW2 (Fig. 1). Longitudinal sections also showed differences in fibrillin-1 expression

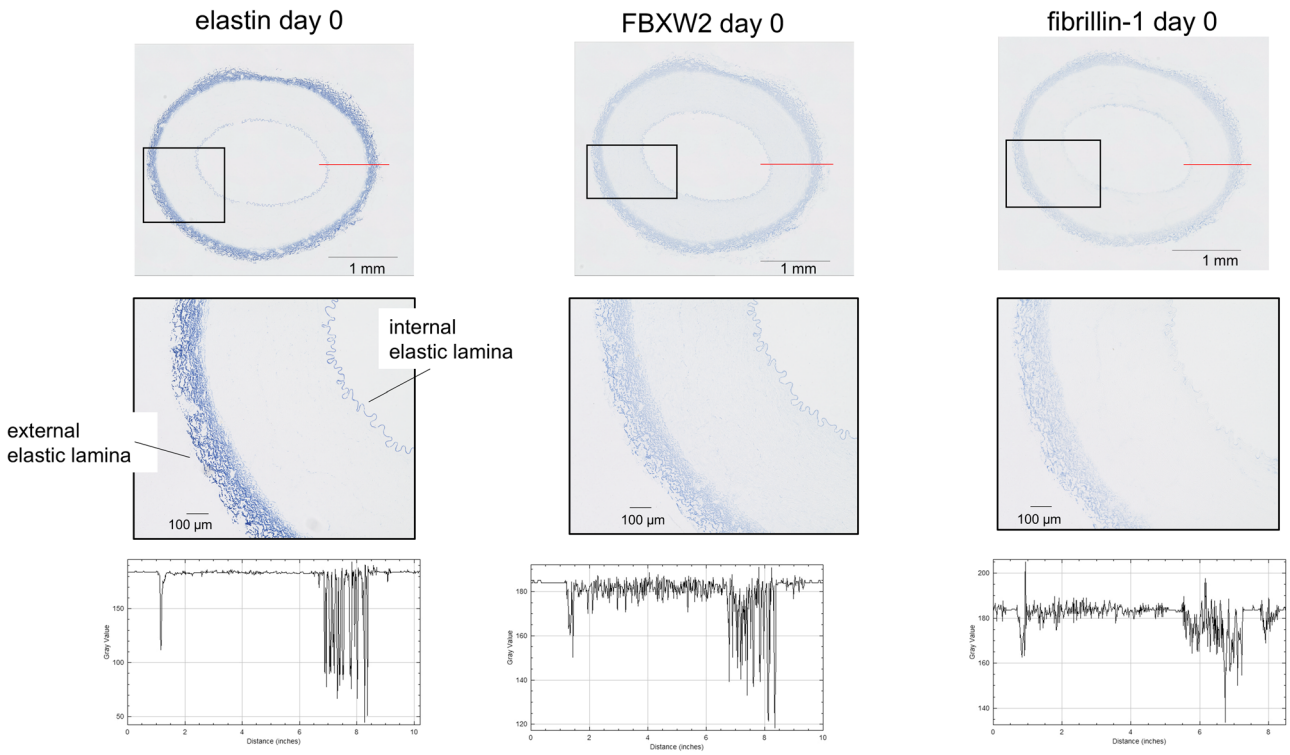

Fig. 1 Serial cross-sections of elastin, FBXW2, and fibrillin-1 expression in the blood vessels from day 0. Red line: analysis of staining intensity. (middle) Highly magnified images. Elastin and FBXW2 expression was evident in the internal and external elastic laminae. Fibrillin-1 expression was weak in the internal and external elastic laminae, but visible outside the external elastic lamina. (bottom) Analysis of staining intensity using Image J 1.54 f. Cross-sectional analysis of staining intensity reveals elastin and FBXW2 expression in elastic laminae, in which the expression of fibrillin-1 was weaker than that of elastin and FBXW2

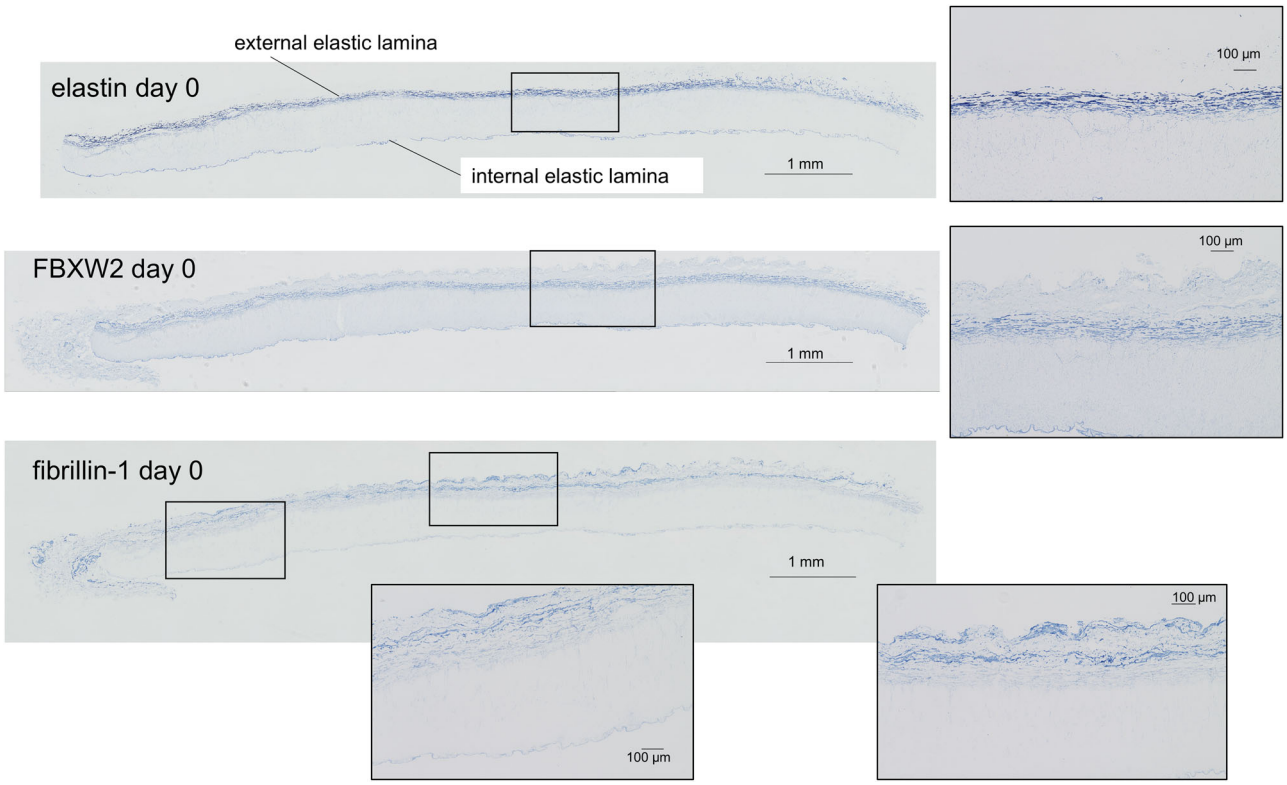

Fig. 2 Serial longitudinal sections of elastin, FBXW2, and fibrillin-1 expression in the blood vessel from day 0. (right, bottom) Highly magnified images. Fibrillin-1 expression was weak in the internal and external elastic laminae, but visible outside the external elastic lamina

from elastin and FBXW2 expression (Fig. 2). Although the cross- and longitudinal sections in Fig. 1 in 2 were obtained from different cows, the results matched well. After 5 weeks of explant culture in two conditions (with and without ascorbic acid), FBXW2 was associated with elastin as well as on day 0, and fibrillin-1 became prominent in the thick adventitia (Figs. 3, 4). The cross- and longitudinal sections in Figs. 3 and 4 were obtained from the same cows as those in Fig. 1 (cross-sections) and 2 (longitudinal sections). fibrillin-1 expression was clearly noted in elastic fiber cleavage regions and the adventitia. Serial sections of immunohistochemistry and HE staining revealed that 5 weeks of explant culture resulted in the occlusion of some of the blood vessel lumens, i.e., 4 out of 10 vessels from 5 cows with 2 conditions (Figs. 5, 6, 7, 8). No occlusion was observed on day 0 (Figs. 1, 5a, 7, 8). In the condition with ascorbic acid, cleavage of elastic fiber and narrow lumen were observed and α-SMA was weakly expressed in the intima layer, and the tissue containing fibrillin-1 narrowed the lumen (Fig. 5b). In explant culture without ascorbic acid, tissue containing fibrillin-1 passed from the intima layer, through the media, to the adventitia where elastic fiber was cleaved and the expression of α-SMA was weak (Fig. 5c). Figure 6 shows double immunohistochemistry results of FBXW2 and fibrillin-1 using the same artery showed in Fig. 9 (lower images). High-magnification images in Fig. S2a–d show the localization of fibrillin-1 to the internal and external elastic lamina. Similarly, serial sections with single immunostaining, double immunohistochemistry showed clear contrast between FBXW2 and fibrillin-1 and revealed that fibrillin-1 expressed inside internal elastic lamina and outside external lamina.

Figure 7 showed HE staining of artery (day 0, 5 weeks after culture with ascorbic acid, 5weeks after culture without ascorbic acid). HE staining revealed that at day 0, necrosis was not observed, while 5 weeks after culture with and without ascorbic acid, weak stained and small nuclei in media were observed. Figure 8 shows fibrillin-1 immunostaining using sections from the same cow in Fig. 7 in order to compare with HE staining. Fibrillin-1 was positive in the adventitia layer and around the intima layer. Figure 9 shows a comparison between fibrillin-1 and α-SMA expression, revealing a correlation between weak α-SMA expression and fibrillin-1. Figure 10 shows three negative control of anti- mouse, goat, and rabbit primary antibodies. No staining in three negative controls. α-SMA immunohistochemistry analysis was conducted on tissues from the cows used for sectioning in Figs. 1, 2 (Fig. S3). Because sections of artery shown in Fig. 2 are longitudinal sections and Figs. 1 and 2 did not show a-SMA expression, two

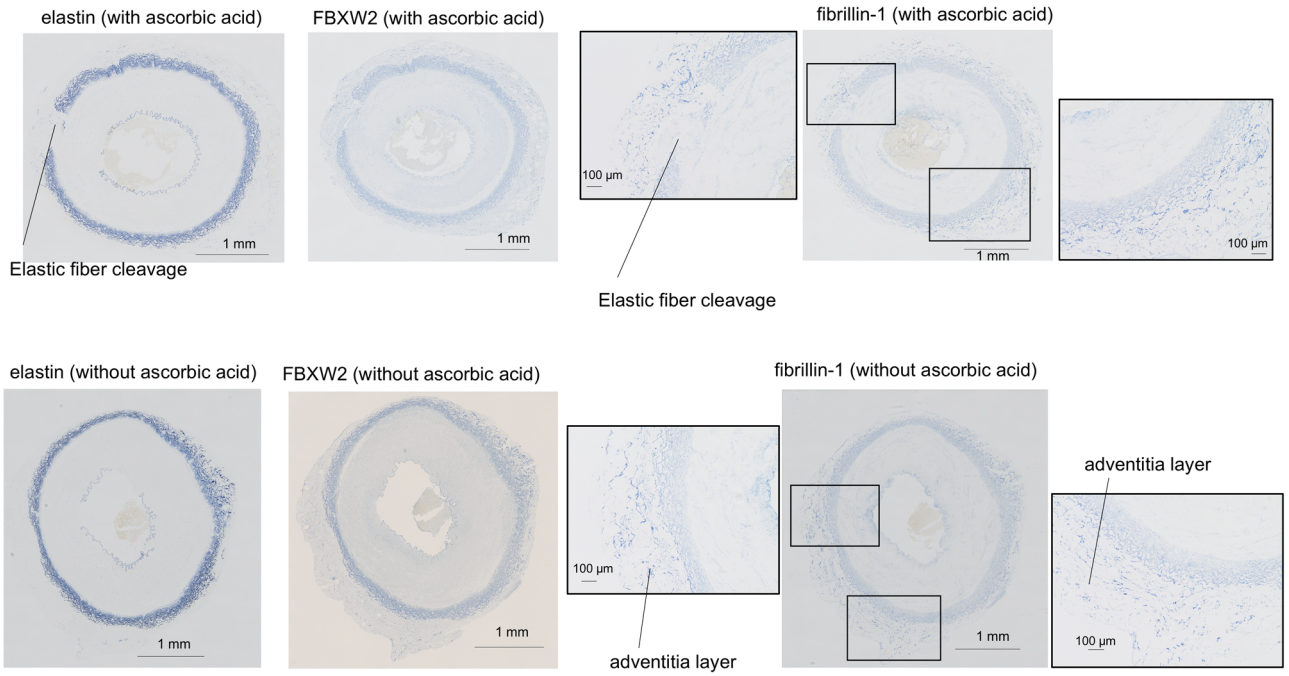

Fig. 3 Serial cross-sections of elastin, FBXW2, and fibrillin-1 expression in the blood vessels at week 5. (top) With ascorbic acid; (bottom) without ascorbic acid. Fibrillin-1-rich adventitia becomes thick. Black-lined squares are high-magnification fibrillin-1 images

cross-sections of a-SMA expression are shown in Fig. S3. Only cross-sections can reveal occlusion of blood vessels. Although occlusion of blood vessels was not observed in all artery, α-SMA expression weakened after 5 weeks without ascorbic acid in all four arteries (Figs. 5, 6, Fig. S3).

Quantitative analysis is displayed in Fig. S4a–f and excel file (MeasureResultsFig. S4). To reduce background staining, positive staining area (colored with light purple, red, and green) was selected as the extracted area. Cross-sections in Fig. S4a–c and longitudinal sections in Fig. S4d, e revealed that area of fibrillin-1 was smaller than that of elastin and FBXW2 because fibrillin-1 expression was weak. Longitudinal sections in Fig. S4f revealed that the fibrillin-1 area was larger than that in elastin and FBXW2, with a thick fibrillin-1 rich layer.

Discussion

Components of Elastic Fiber

This study showed that in elastic fibers of the vascular system, FBXW2 was associated with elastin from day 0 to week 5 with and without ascorbic acid, whereas fibrillin-1 was disassociated (Figs. 1–5). Results of double immunohistochemistry supported those obtained based on the serial sections in this study (Fig. 6). Because the locations of elastin and FBXW2 was similar, we observed overlapping colors in double staining of elastin and FBXW2 [29]. Although the antibodies against both elastin and fibrillin-1 are mouse monoclonal antibodies, double immunohistochemistry of elastin and fibrillin-1 cannot be performed; therefore, the position of elastin and fibrillin-1 comparative to that of FBXW2 can be useful. After 5 weeks of explant culture of periosteum without ascorbic acid, the dissociation of FBXW2 from elastin was observed [31]. The relationship between elastin and FBXW2 in blood vessels is different from that in periosteum. The author presumed that elastin, FBXW2, and fibrillin-1 would be components of elastic fibers, and three components were expressed in internal and external elastic laminae. However, fibrillin-1 was expressed in a region different from where elastin and FBXW2 were expressed and the findings are by-products. However, the role of FBXW2 in elastic fibers is still unknown, although this study showed that FBXW2 (instead of fibrillin-1) was expressed in the elastic lamina close to elastin after 5-week culture. Fig. S5 shows fluorescent double immunohistochemistry of FBXW2 and fibrillin-1 in periosteum, with arrows indicating few fibrillin-1 layer. The region of fibrillin-1 expression was not the same as that of FBXW2 in blood vessel and periosteum (Figs. 6, S5). Fibrillin-1 expression was fewer in periosteum. In the periosteum, FBXW2 was associated with osteocalcin expression [30]. The amount of fibrillin-1 may affect osteocalcin expression on FBXW2. Elastin of artery are known to show autofluorescence [33]. In bovine artery, fluorescent double immunohistochemistry of FBXW2 and fibrillin-1 resulted in elastin autofluorescence in especially in internal elastic laminae (data not shown). Therefore,

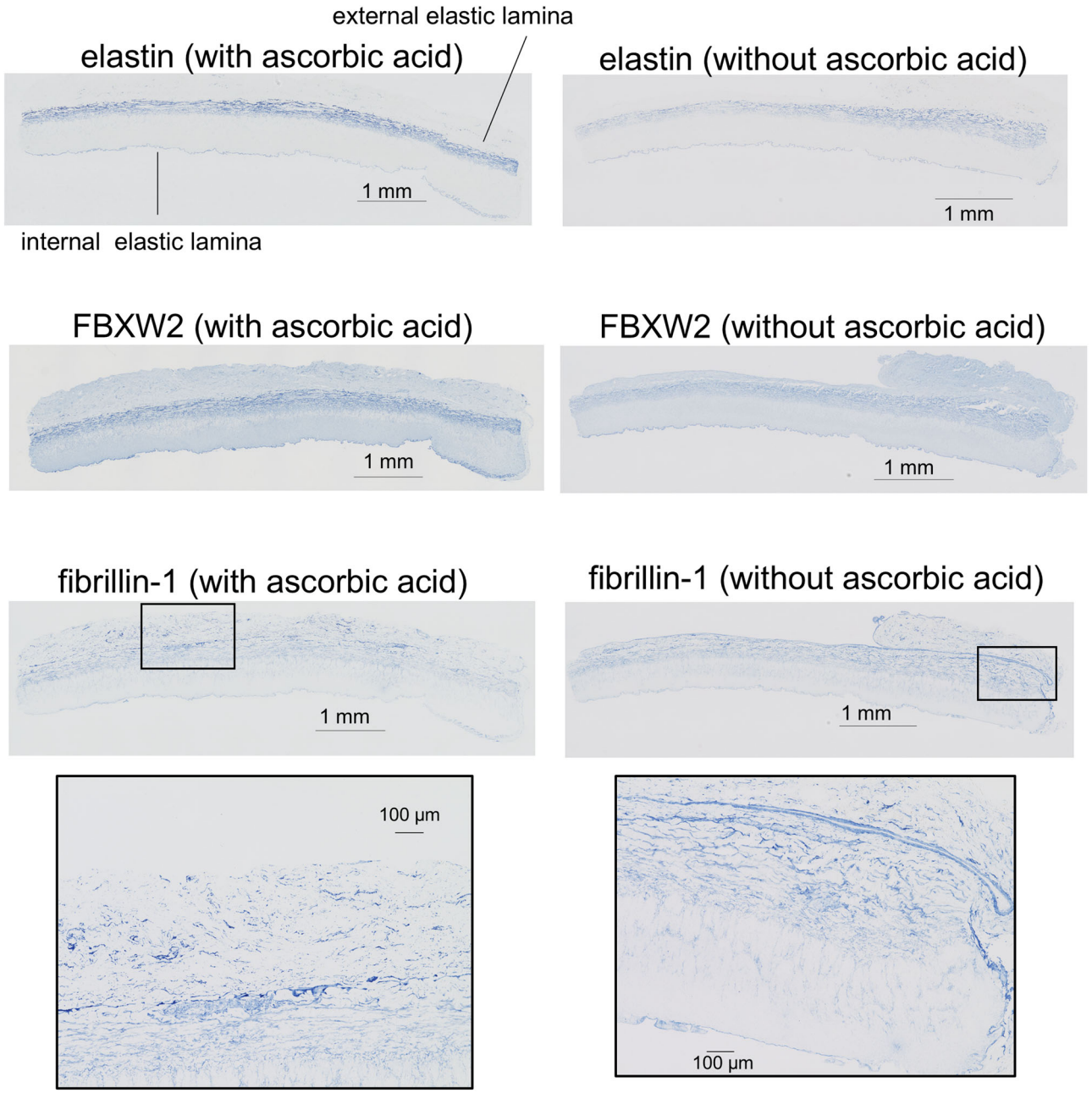

Fig. 4 Serial longitudinal sections of elastin, FBXW2, and fibrillin-1 expression in the blood vessels at week 5. (left) With ascorbic acid; (right) without ascorbic acid. Fibrillin-1-rich adventitia becomes thick. (bottom) Black-lined squares are high-magnification fibrillin-1 images

serial sections and alkaline phosphatase-conjugated secondary antibodies that does not show autofluorescence were used.

Staining of Elastic Fiber

Resorcin-fuchsin staining was used for identifying elastic fibers and oxytalan fibers [34–36]. In 1997, Augsburger [34] reported oxytalan fibers in canine urethra. In 2002, Porto et al. [35] showed the presence of delicate oxytalan fibers in saphenous varicose veins. In 2005, Xavier-Vidal [36] reported oxytalan fibers in myocardium. However, resorcin-fuchsin staining cannot distinguish elastin from fibrillin-1; previous studies might have determined that thin and delicate fibers made from elastin were oxytalan fibers. In this study, it was found that FBXW2 was expressed with elastin rather than that with fibrillin-1 (a well-known component of elastic fiber). Elastica van Gieson (EVG) staining is also used for studying elastic fibers [37]. Figure S6 shows EVG staining of bovine blood vessels; however, the internal

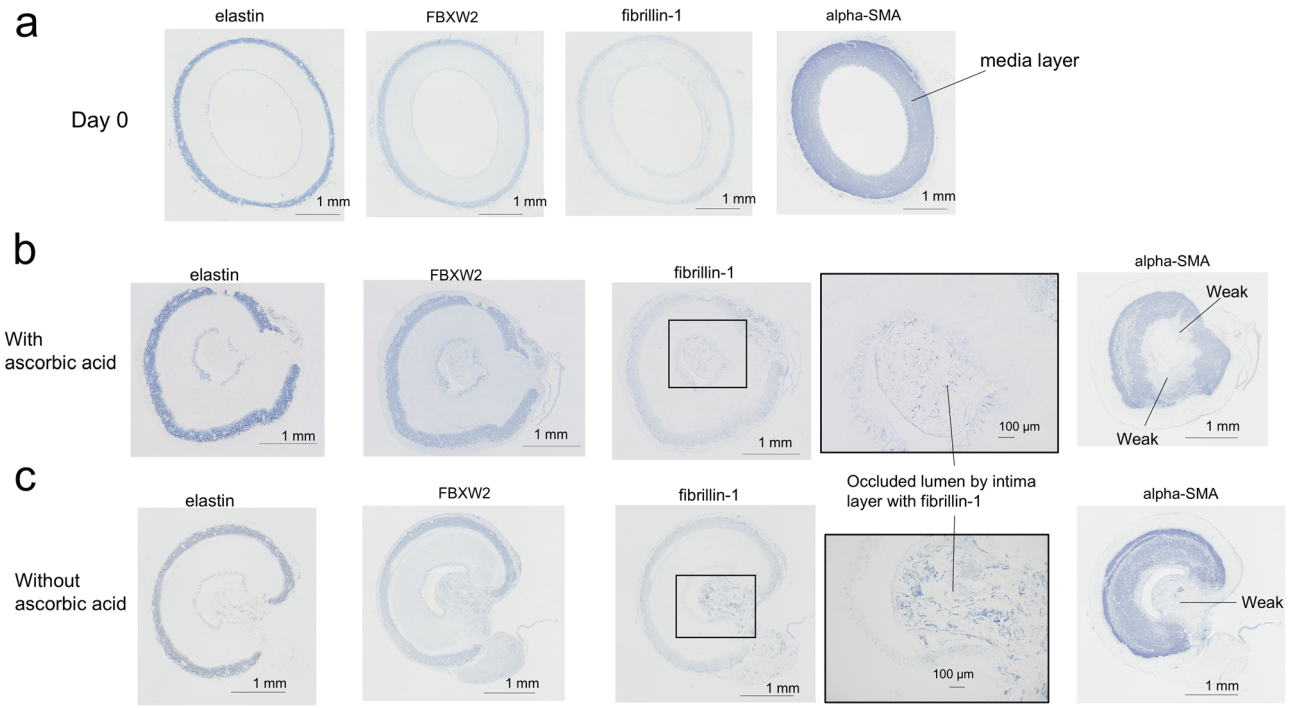

Fig. 5 Serial sections were examined for elastin, FBXW2, fibrillin-1, and α-SMA expression. **a** Day 0. **b** Five-week culture with ascorbic acid. **c** Five-week culture without ascorbic acid. Fibrillin-1-rich tissue blocked the lumen of blood vessels and α-SMA expression was weak in this region. Black-lined squares are high-magnification fibrillin-1 images

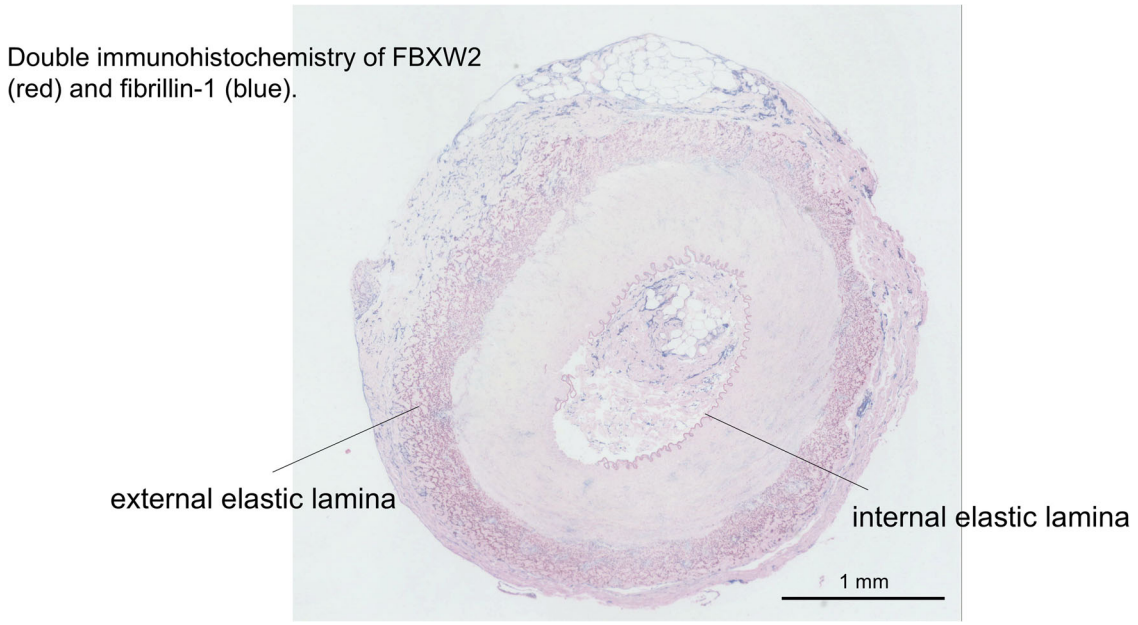

Fig. 6 Double immunohistochemistry of FBXW2 (red) and fibrillin-1 (blue) in blood vessels. Without ascorbic acid

elastic laminae were vaguely stained. In 2010, Kamino et al. [37] showed elastic fiber patterns in melanoma with EVG staining and elastin immunostaining and reported that elastin immunostaining was superior to that by EVG. However, they did not perform fibrillin-1 immunostaining and could not detect oxytalan fibers.

Fibrillin-1 and Elastic Fiber

Although fibrillin-1 is a known component of elastic fibers [13, 38], the current study showed that elastin and fibrillin-1 were not expressed in exactly the same region of elastic lamina (Figs. 1, 2). This result could be attributed to the small

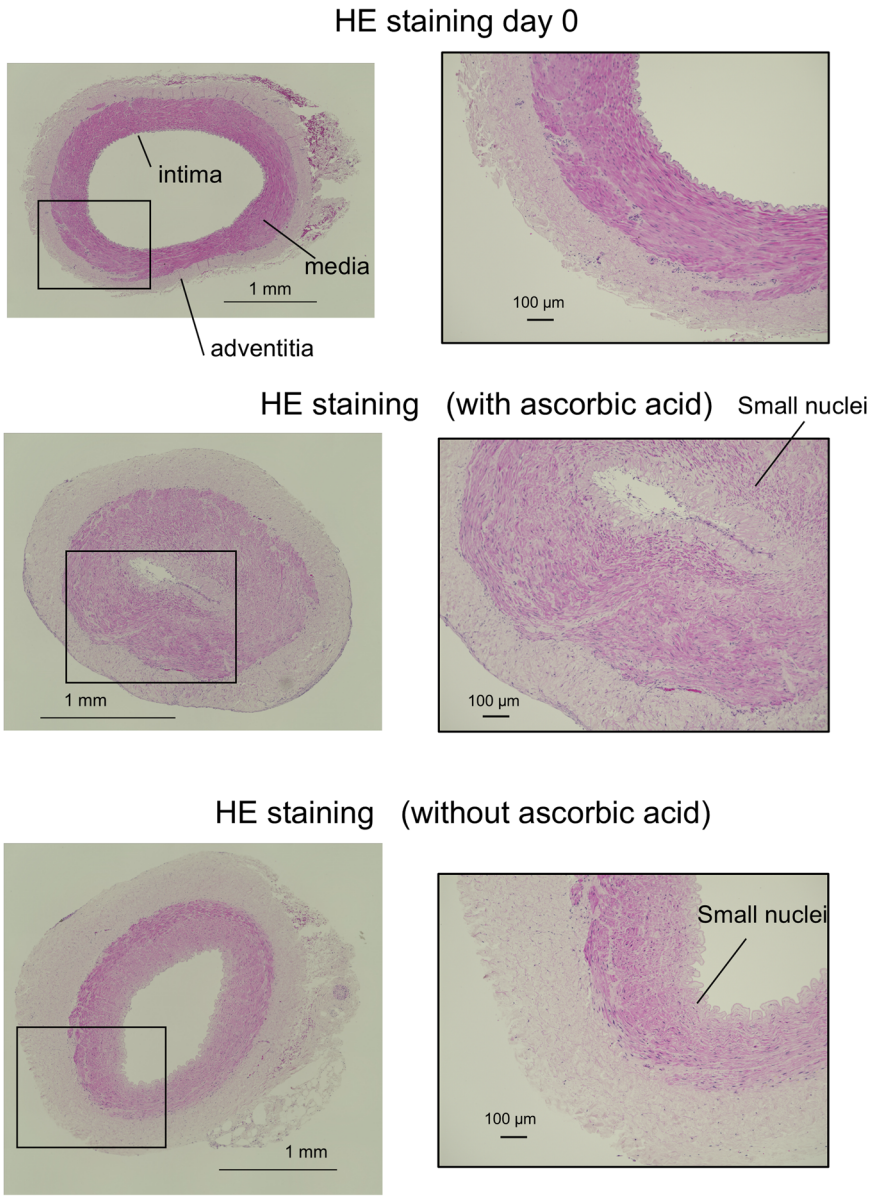

Fig. 7 Hematoxylin Eosin staining. 5 weeks after culture, weak stained media and small nuclei in media were observed. Black-lined squares are high-magnification of left images

amount of fibrillin-1 in the elastic lamina. Another reason may be elastic fiber coverage by elastin. Steijns et al. [39] revealed that fibrillin-1 and elastic fibers were expressed in the vessel wall of tunica media; however, an antibody against elastin was not used in this study; furthermore, they used small vessels. Histological observations revealed that the fibrillin-1-positive microfibrils in the thyroepiglottic ligament were probably oxytalan fibers, which were inconsistent with the shapes of elastic fibers [16]. Sugarawa et al. [40] reported that in the periodontal ligament of the rat molar, fibrillin-2 and microfibril-associated glycoprotein-1 are elastin-free microfibrils. Wang et al. [41] investigated a complex of electrospun tropoelastin and smooth muscle cells, fibroblasts, and pericytes and showed that the fiber shapes of elastin and fibrillin-1 are different.

In this study, the adventitia with fibrillin-1-rich layer thickened over the 5-week culture period. It is possible that the fibrillin-1-rich original layer on day 0 (Figs. 1 and 2) grew along within the adventitia after explant culture. Elastin maintained the shape of elastic lamina even after cleavage, whereas the fibrillin-1-rich layer moved from the elastic lamina (Figs. 3, 4).

More than 30 microfibrillar proteins are known to be associated with elastic fibers [27]. Microfibrillar-associated protein 4 is expressed in elastin-rich tissue and is known to regulate elastic fiber arrangement [28]. The current study focused on elastin, the well-known component of elastic fibers in elastic lamina of blood vessels, and fibrillin-1 (another component of elastic fiber) associated with aortic aneurysm (one of the symptoms of Marfan syndrome) [24].

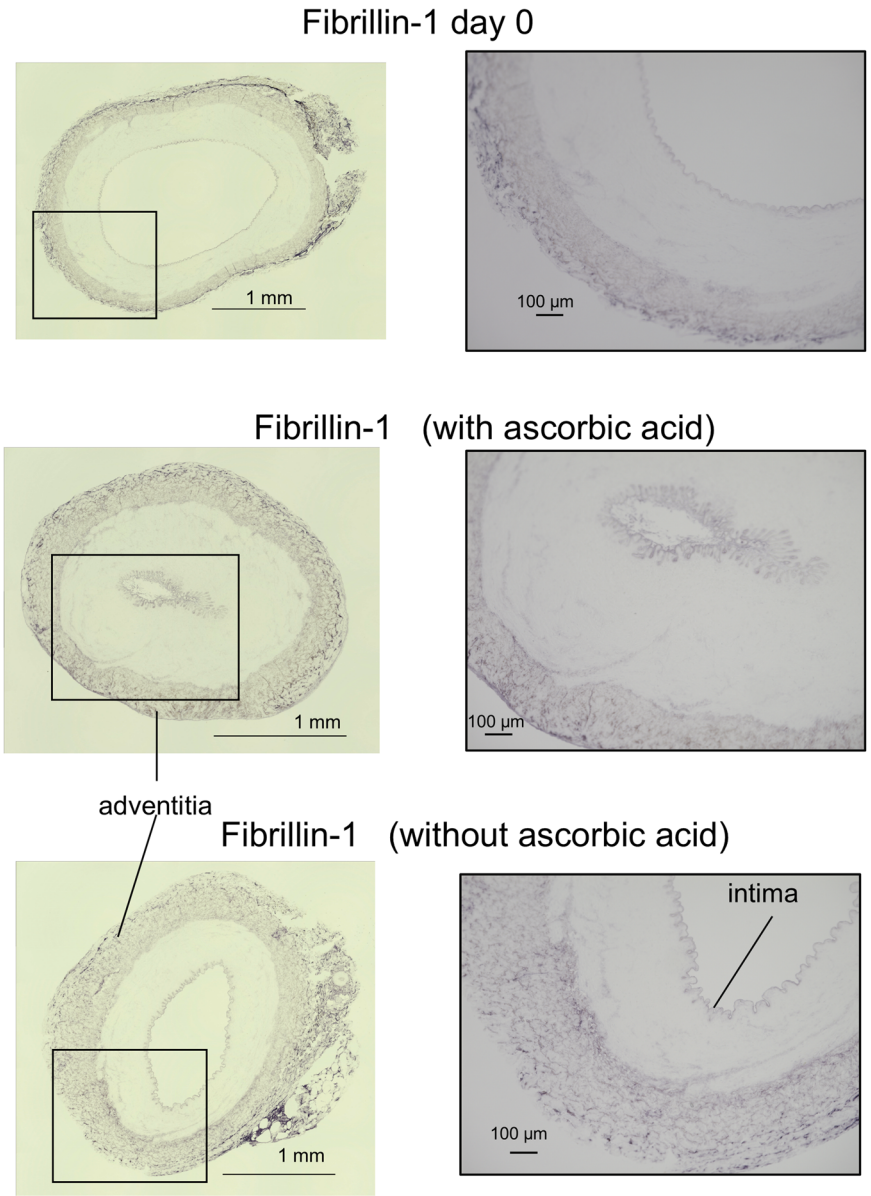

Fig. 8 Fibrillin-1 immunostaining. Sections from the same cow in Fig. 7 were used in order to compare with HE staining. Black-lined squares are high-magnification of left images

In this study, microfibers made from fibrillin-1 were present in other regions of elastin. These results showed that oxytalan fibers (made from fibrillin-1) are present around the elastic lamina of blood vessels.

Composer of Blood Vessel

The investigated blood vessels (Fig. S1) are arteries because the arterial walls of smooth muscle cell layers are thick compared to those of the vein walls [42]. Figure S7 shows elastin immunostaining of an artery and the adjacent vein. Compared to those of the artery, the vein does not have internal elastic laminae and the blood vessel lumen is small (Fig. S7). In a previous study, the amount of fibrillin correlated with SMC apoptosis in primary varicose veins and the authors compared two groups (under 50 and over 50) but did not use either serial sections or antibodies for elastin [43]. In the current study, lumen was narrowed by fibrillin-1-rich tissue and weak α-SMA expression occurred simultaneously (Figs. 5, 9). Therefore, further studies are needed to clarify the relationship between lumen occlusion and reduction in α-SMA expression. Aortic injuries caused by stent graft stress sometimes caused medial necrosis and stretching of elastic fiber [44]. Yang et al. [45] reported that hypercalcemia and hyperglycemia altered tropoelastin structure by changing the hydrogen-bonding pattern and mechanical function. Ramamurthi et al. [4–8] investigated the relationship between the development of abdominal aortic aneurysm and elastic matrix degradation and nitric oxide signaling, stress-activated

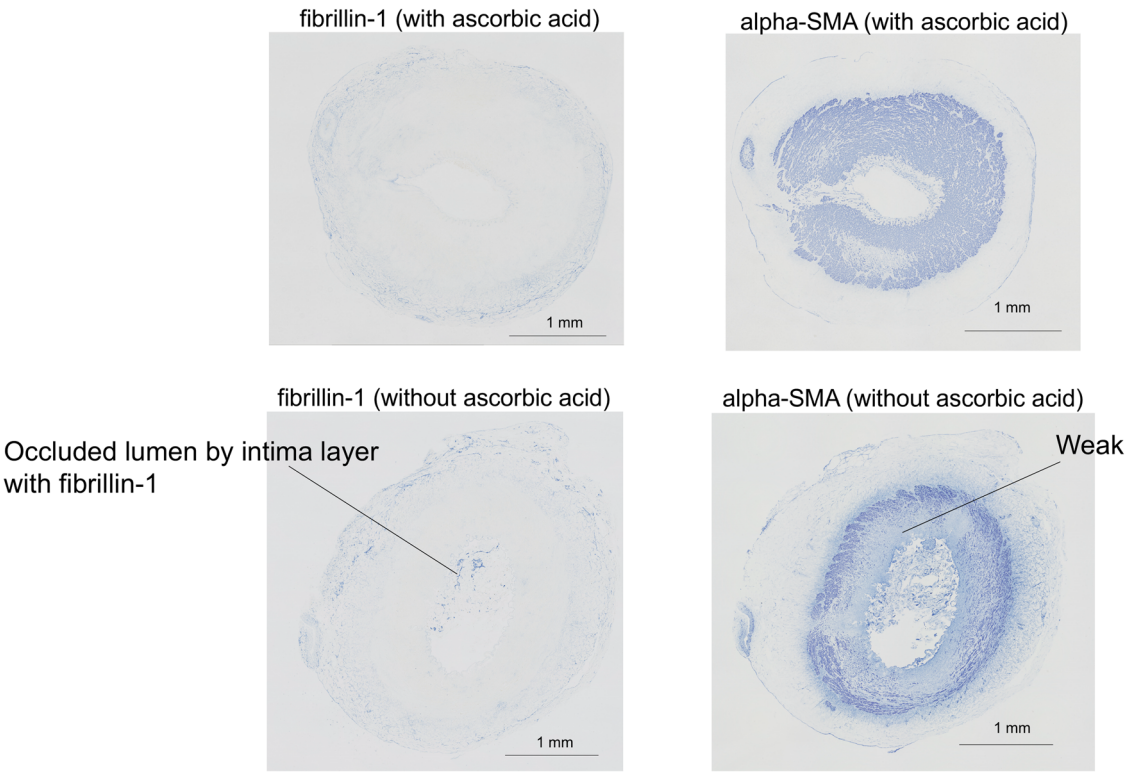

Fig. 9 Five-week culture; comparison of fibrillin-1 and α-SMA expression. The sections are not serial sections but are from the same cows. Without ascorbic acid, fibrillin-1-rich tissue occluded the lumen and α-SMA expression became weak. A section is from the same cow in Fig. 6

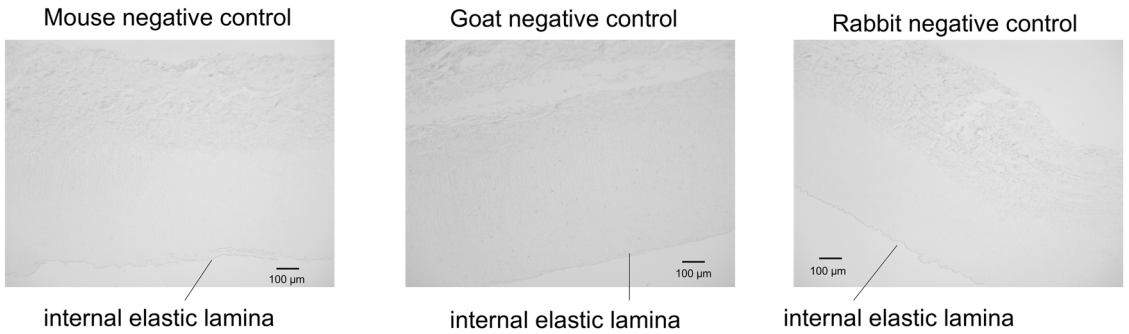

Fig. 10 Three negative controls for anti-mouse, anti-goat, and anti-rabbit primary antibodies

protein kinase JNK-2 expression, and epidermal growth factor receptor activity of SMCs. The correlation between NOTCH3 expression and elastic fiber dispersion in the infrarenal aorta was reported [19]. The fibrillin-1-regulated microRNA miR-122 is related to inflammatory pathways in SMC [11]. The association between mesenchymal stem cells (MSC) and elastic fibers was investigated [20]. Jeong et al. [46] reported that the fibrillin-elastin fragment enhanced MSC differentiation into SMCs. González-Pérez et al. [47] formed biomimetic vascular replicas with elastin-like recombinamer hydrogel.

Ascorbic Acid Conditions

Cows can synthesize ascorbic acid in the liver; however, in the current study, a need for supplementation of ascorbic acid into the culture medium existed because the cows used in this study were dead. Antioxidant vitamins and ascorbic acid were investigated with respect to vascular inflammation using vascular smooth muscle cells or vascular endothelial cells [48–50]. Cell uptake of ascorbic acid inhibits the inflammatory response caused by oxidant stress [48, 49]. Anti-atherogenic compounds involving ascorbic

acid inhibit the responses of vascular smooth muscle cells [51]. Aortic SMCs cultured with ascorbic acid decrease calcium accumulation [52]. Here, vascular inflammation of vascular smooth muscle cells may have caused shrinkage of the media layer of blood vessels.

The culture media conditions of ascorbic acid were important factors in the current study. The diffusion of FBXW2 from elastin was observed at 5-week explant culture without ascorbic acid in periosteum; however, FBXW2 could not keep the shape of fibers [31]. In blood vessels, disassembly and denaturing of elastic fibers may cause a narrow vessel lumen at the internal elastic laminae or collapse of blood vessel shape at the external elastic laminae. Therefore, similar to the that in periosteum, two conditions of ascorbic acid were investigated. However, in this study, elastin and FBXW2 maintained their fiber shapes in the blood vessels with and without ascorbic acid. This may be to maintain the shape of the elastic laminae. The presence of ascorbic acid affects the 2D and 3D structures of periosteum-derived cells [53]. However, in this study, wall remodeling of the blood vessels was observed with and without ascorbic acid.

3D In Vitro Vascular Model

Studies using in vitro models of blood vessels were performed previously. For example, vascular smooth muscle, endothelial, and human induced pluripotent stem cells were used in 2D and 3D models [54–58]. After arrest of blood flow, vascular smooth muscle cells can be isolated from aortic explants [56]. Blood vessel organoids attracted attention [57, 58]; however, these organoids do not have elastic laminae. Kumar and Varghese [59] investigated tumor-on-a-chip models for tumor angiogenesis. Osaki et al. [60] investigated angiogenesis using a tissue engineering method in a 3D in vitro model. However, these previous studies did not represent the native vascular structure. The vascular model used in the current study did not require special equipment, except for a CO_2 incubator.

Limitation of the Study

A limitation of our study was related to the culture and arteriectomy conditions. Fetcher et al. [10] reported elastin loss and microcalcification in thoracic aortopathy samples from patients with severe thoracic aortic aneurysm. In the periosteum, osteocalcin was expressed, whereas in blood vessels, it was not expressed [29, 30]. This difference in expression may be related to calcification. FBXW2 binds to the muscle segment homeobox 2 [61], which was investigated with aortic calcification [62–64]. In future studies, calcification or osteocalcin expression in blood vessels should be investigated under various culture media conditions.

The conditions under which arteriectomy is performed must also be investigated. Furthermore, we did not consider the connective tissue around the blood vessels. Application of different tension levels to remove blood vessels from connective tissue may affect the degree of occlusion of blood vessels.

More than five bovine legs were used in this study. However, the author excluded arteries with veins (Fig. S7) or those not meeting three conditions (day 0, with ascorbic acid, without ascorbic acid). Thus, the number of arteries used herein (five arteries) is a significant limitation to the repeatability of the study.

Here, the expression of a well-known component of elastic fibers, fibrillin-1, differed from that of elastin and FBXW2 from day 0 to 5 weeks. Unlike in the periosteum, FBXW2 in the blood vessels was not separated from elastin in the condition without ascorbic acid. The difference between the periosteum and the blood vessels, (about the separation of FBXW2 from elastin) may result in different osteocalcin expression. The different amount of fibrillin-1 between the periosteum and the blood vessels also may affect osteocalcin expression. This in vitro 3D model is suitable for clarifying the mechanism of lumen occlusion or calcification of elastic fibers because this model includes all blood vessel structures with the intima, media, adventitia, vascular endothelial cells, and smooth muscle cells. Upon arteriectomy, the presence of connective tissue around the blood vessels and differences in tension to remove blood vessels from this tissue can be better studied with the aid of this model. The findings in this study state that investigation of blood circulation can potentially prevent artery disease in human vascular biology.

Data availability

A pre-print version of this article is available on bioRxiv (https://doi.org/10.1101/2024.02.06.579234). The data that support the findings of this study are openly available at Biomimetics in Fig. 2a, b 2022-12; (https://doi.org/10.3390/biomimetics8010007), reference number [29].

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s12013-024-01647-5.

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Author contributions M.A. designed research, performed immunohistochemistry, analyzed data, and wrote the paper. There are no co-authors.

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Compliance with ethical standards

Conflict of interest The author declares no conflict of interest.

Research involving Animal The animal study protocol was approved by the Institutional Review Board of the Osaka Dental University Regulations on Animal Care and Use (protocol code 23-02007 and date of approval: March 13, 2023).

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