JSHC
Japan Society of
Histochemistry and Cytochemistry
Established 1959

Acta Histochem. Cytochem. 49 (1): 29–36, 2016
doi: 10.1267/ahc.15028

Histopathological Characteristics of Post-inflamed Coronary Arteries in Kawasaki Disease-like Vasculitis of Rabbits

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Received August 12, 2015; accepted December 25, 2015; published online January 22, 2016

Kawasaki disease (KD) is a systemic vasculitis in infants that develops predominantly in the coronary arteries. Despite the clinically transient nature of active inflammation in childhood albeit rare complications (e.g., coronary artery aneurysm), KD has recently been suggested to increase the incidence of ischemic heart diseases in young adulthood. However, little is known about the histopathology of the coronary artery long after development of the acute KD vasculitis. To address this, we conducted histological studies of rabbit coronary arteries in adolescent phase after induction of the KD-like vasculitis induced by horse serum administration. After a transmural infiltration of inflammatory cells in acute phase at day 7, the artery exhibited a gradual decrease in the number of inflammatory cells and thickening of the intima during the chronic phase up to day 90, where proteoglycans were distinctly accumulated in the intima with abundant involvement of α-smooth muscle actin (α-SMA)-positive cells, most of which accompanied expression of VCAM-1 and NF-κB. Distinct from classical atherosclerosis, inflammatory cells, e.g., macrophages, were barely detected during the chronic phase. These observations indicate that the KD-like coronary arteritis is followed by intimal thickening via accumulation of proteoglycans and proliferation of α-SMA-positive cells, reflecting aberrant coronary artery remodeling.

Key words: Kawasaki disease, coronary artery, animal model, proteoglycans, vascular smooth muscle cells

I. Introduction

Kawasaki disease (KD) is a systemic pediatric vasculitis, whose etiological mechanism remains elusive [16]. Owing to the applications of intravenous immunoglobulin therapy over the past two decades, striking declines have been established in its mortality during the acute phase and in complications, e.g., dilatation or coronary artery aneurysm [9, 15, 24, 28]. However, growing evidence has accumulated that young adults with a past history of KD have a higher incidence of ischemic heart disease (IHD) as compared with the general population of the same age without a history of KD [30, 39, 43]. Besides the well-known complication of IHD in patients with coronary aneurysm after KD, post-KD young adults with clinically no discernible complications also tend to show thickened intima or stenosis on their coronary arteries, as regarded by vascular remodeling [11, 22, 29, 38]. Thus, past history of KD is currently regarded as a predisposing factor for IHD. However, little is known about the pathogenesis of the post-inflammatory remodeling of the coronary arteries long after development of KD. To prevent the post-KD patients from developing serious IHD, it is an urgent issue to uncover the pathogenesis of the stenotic lesions of coronary arteries long after KD. Because of the limited sort of specimens from patients

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with KD, studies on KD have been performed predominantly by using animal models, e.g., mouse and rabbit, which have provided valuable information on pathologic aspects of the disease [10, 12, 18, 23, 32, 34]. To the best of our knowledge, however, there has been no experimental study as to the post-inflammatory changes in KD with animal models, albeit there is one report on the atherogenesis induced by high-fat diet in a mouse KD model [7]. In the present study, we performed histological analysis of coronary arteries in a KD-like vasculitis of rabbits as established by Onouchi *et al.* [32] at the acute (~day 7) and chronic (days 60 and 90) time phases. We found that post-inflammatory remodeling of coronary arteries is associated with predominant accumulation of proteoglycans in the lesion by proliferation of activated vascular smooth muscle cells, which may lead to progression to coronary arteriosclerosis.

II. Materials and Methods

Animal model

The investigation conformed with *the Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) under approval of the Animal Care Committee of Kyoto Prefectural University of Medicine. We used a KD-like, allergic vasculitis model in rabbits as established by Onouchi *et al.* [32] during a weaning phase. Horse serum (hereafter, HS) (cat.No.16050130, Life Technologies) was applied to 5-week-old Japanese white male rabbits weighing ~700 g by intravenous injection (10 ml/kg body weight), twice at two-week intervals. Freund's complete chemical adjuvant (0.5 ml with an equivalent volume of horse serum) was subcutaneously applied 1 week before the first injection of HS. The hearts were excised from the rabbits under generalized anesthesia by intravenous injection into the ear vein with pentobarbital sodium (~100 mg/kg body weight) during acute vasculitis phases at days 1, 3, 5, and 7 and healing phase at day 21, and chronic phases at days 60 and 90. As a control, hearts obtained from rabbits after injections of saline (10 ml/kg body weight) were examined at days identical to those in vasculitis models.

Histological analyses

After fixation of the hearts with coronary perfusion via the ascending aorta with 4% paraformaldehyde in phosphate-buffered solution (PBS) for 3 min followed by immersion for more than half a day, the main trunks of the right and left coronary arteries were excised and embedded in paraffin. Serial slices were obtained from the paraffin-embedded specimens at 4-μm thickness. We performed hematoxylin and eosin (HE) staining and Elastica van Gieson (EVG) staining for histological analysis. Proteoglycans were detected by alcian blue (pH 2.5) stain. We determined the intima as the tissue inside the internal elastic lamina and the media as the tissue between the internal and external elastic laminas on EVG-stained slides. Samples of near peripheral branches or oblique sections were excluded from the analysis. We measured the thickness of the intima, media and diameter of the coronary arteries having diameters larger than 400 μm and calculated the intima/diameter ratio and media/diameter ratio of the arteries. Within the individual arteries, measurements were made at the site in which the intima was most prominently thickened. The specimens with vertical section were excluded from analysis. For immunohistochemistry, deparaffinized sections were submitted for reaction with each antibody after the blocking procedure with 1% bovine serum albumin in PBS. Mouse monoclonal antibody for α-smooth muscle cell actin (α-SMA) (1:1000; Sigma-Aldrich) was applied to the samples for the first antibody, and subsequently alkaline phosphatase-combined goat anti mouse antibody was used for visualization with addition of New Fuchsin dye. Anti-vascular cell adhesion molecule-1 (VCAM-1, mouse monoclonal 1:100; NOVUS Biologicals), anti-nuclear factor-κB (NF-κB; mouse monoclonal, 1:200; Santa Cruz), and anti-macrophage (RAM-11, mouse monoclonal, 1:100; DAKO) antibodies were secondarily applied with hematoxylin counterstain. For the VCAM-1 immunohistochemistry antigen retrieval was performed in 10 mM citrate buffer (pH 6.0), and for NF-κB, in 10 mM Tris-HCl buffer containing 1 mM EDTA (pH 9.0) in a pre-heated water bath at 98°C for 30 min. We visualized signals by applying horseradish peroxidase-conjugated goat anti-mouse antibody with DAB.

Quantitative analysis

Histological images were acquired with an Olympus BX51 microscope with objective lenses of Plan Apo (numerical apertures of 0.4 for ×10, 0.75 for ×20, and 0.9 for ×40) and with a color CCD camera (DFC320, Leica) for digital micrographs. α-SMA and VCAM-1 double positive cells were determined by quantitative immunohistochemistry. The ratio (%) of double positive cells was calculated for all α-SMA-positive cells. The number and density of the nuclei of α-SMA-positive cells, contained within the 100-μm width of cross sectioned medial wall, were counted (at day 90). For quantification of proteoglycans, digital micrographs of specimens stained by alcian blue were processed by binarization of the images with ImageJ software (NIH). Thereafter, measurements were made for percentage of the area occupied by proteoglycan over the total vessel wall area.

With digital micrographs of specimens stained for NF-κB, the positive stain was determined by the number of positive nuclei and cytoplasm. Quantitative data are expressed as the box plots. Statistical analyses were conducted with SPSS software. Comparisons between the two groups were done with Mann-Whitney U test, and those among the four groups were done with Kruskal-Wallis analysis. For all analysis, $p<0.05$ was accepted as statistically significant.

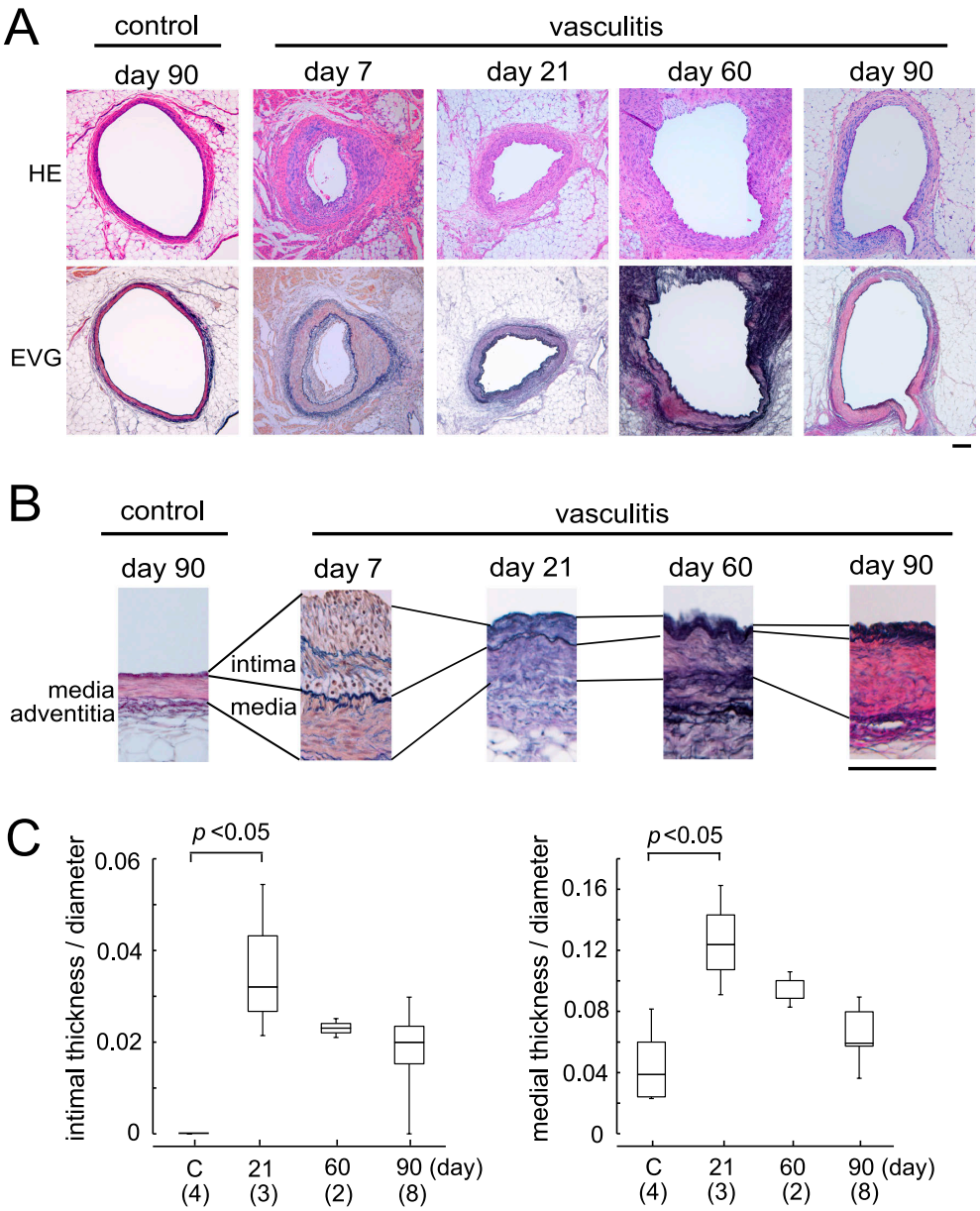

Fig. 1. The time-dependent histological changes in the inflamed and post-inflamed coronary arteries. (**A**) HE and EVG staining images of coronary arteries in the control and vasculitis rabbits. Bar = 100 μm. (**B**) High power images of the local areas in (**A**). (**C**) Graphs showing relative thickness of the intima and media over the vessel diameter shown as box plots. The number of animals used for the quantification is indicated in parentheses.

III. Results

Histological change in the coronary arteries of the rabbit model for KD

We first examined histological changes in the coronary arteries of the HS-treated rabbits at different time points. The HE-stained samples revealed a marked infiltration of inflammatory cells in the intima and media at days 3 and 7 after administration of the horse serum (Supplementary Fig. 1A and Fig. 1A). In accordance with the inflammation, EVG-stained samples revealed a remarkable thickening of the intima with irregular aligment of internal electic lamina (Fig. 1B). Consistent with the acute inflammatory changes, the intima and media were significantly increased in thickness at day 7, indicating the inflammation-associated swelling of the coronary arterial wall (Fig. 1C). Such inflammatory changes waned with time, and eventually inflammatory cell infiltration disappeared nearly completely at day 21 after application of HS as shown in Fig. 1B. Macrophages identified by RAM-11 immunohistochemistry at day 7 had disappeared at day 90 (Supplementary Fig. 1B). In sharp contrast, coronary arteries of the age-matched rabbits without HS application exhibited neither the inflammatory cells nor medial thickening (the leftmost pictures in Fig. 1A & 1B and Supplementary Fig. 1A). The thickening of the intima and media, grad-

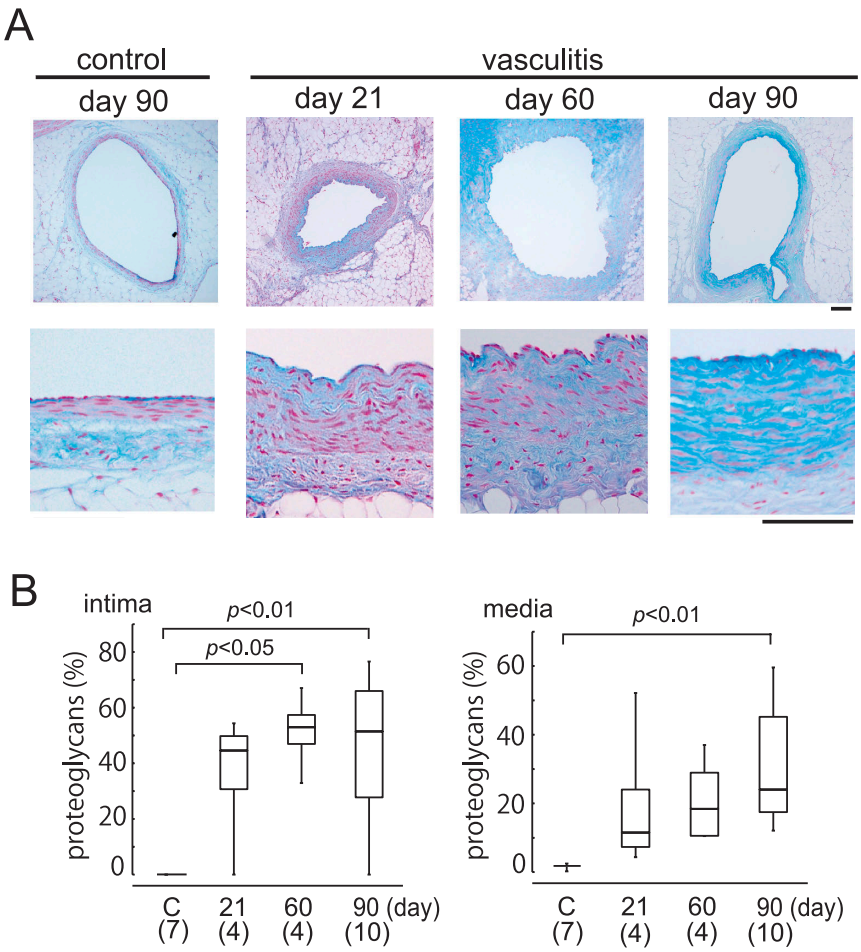

Fig. 2. Accumulation of the proteoglycans in inflamed and post-inflamed coronary arteries. (**A**) Alcian blue staining of coronary arteries in control and in vasculitis. Bars = 100 μm. In the lower panel high-power images of the local area in the upper panel. (**B**) Percent population of Alcian blue-stained areas in the intima (left panel) and media (right panel) are shown as box plots. The number of animals used for the quantification is indicated in parentheses.

ually decreasing with time, was not completely restored to the control levels even at day 90 after HS administration, suggesting persistent post-inflammatory tissue remodeling of the coronary arteries after resolution of the acute inflammatory responses.

Accumulation of proteoglycans in post-inflamed coronary arteries

Accumulation of extracellular matrix proteins including proteoglycans is known to be closely relevant to vascular remodeling in atherosclerosis. Alcian blue staining indicated accumulation of proteoglycans in the intima and media (Fig. 2A). The alcian blue-positive materials were strikingly increased in the intima and media of the coronary arteries in HS-treated rabbits in a time-dependent manner (Fig. 2B), whereas such increment was barely detected in the age-matched control rabbits, indicating that the persistent thickening of the post-inflamed coronary artery is associated with the accumulation of proteoglycans in the affected coronary artery.

Sustained activation of vascular smooth muscle cells in the post-inflamed coronary arteries

In the context of possible mechanisms underlying the accumulation of proteoglycans in the post-inflamed coronary arteries, it has been well-documented that activated vascular smooth muscle cells (SMCs) are responsible for excess production of the extracellular matrix components [8]. Indeed, both the number and density of α-SMA-positive cells were significantly increased in both the acute (day 7) and chronic (day 90) phases of the affected coronary artery (Fig. 3A and 3B-a). We further investigated the expression of VCAM-1, a hallmark of activated SMCs [3], in the chronic phase of affected coronary arteries. As shown in Fig. 3B-b, the number of α-SMA-VCAM-1 double positive cells, [α-SMA(+)-VCAM-1(+) cells], were significantly increased in the vascular wall of post-inflamed coronary arteries as well as inflamed ones. Expression of VCAM-1 is known to be governed by NF-κB activation [45], which is recognized by its nuclear translocation [31]. Consistent with this, expression and nuclear translocation of NF-κB were significantly increased in acute inflamed

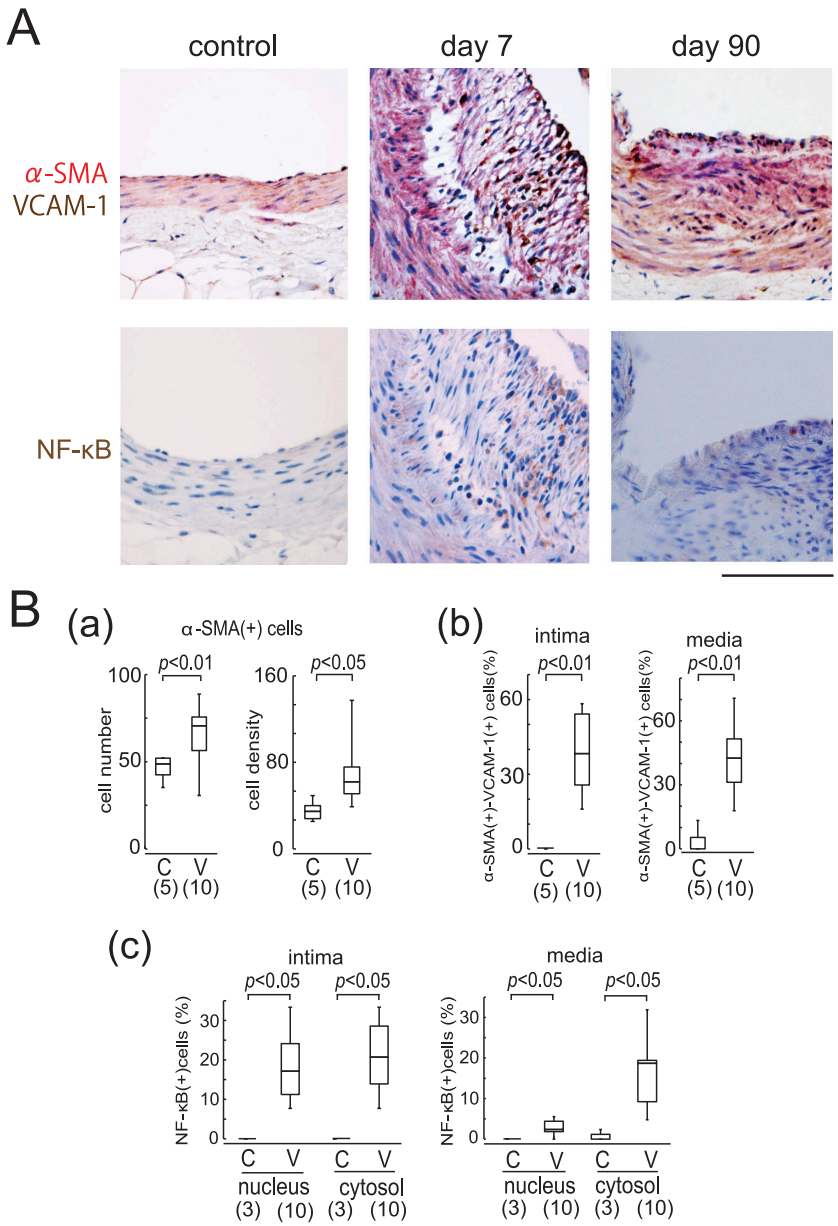

Fig. 3. Immunohistochemistry for α-SMA(+)-VCAM-1(+) and NF-κB(+) cells in the inflamed and post-inflamed coronary arteries. (**A**) Immunohistochemical images of of coronary arteries (100-μm sequential tissue sections) for VCAM-1 (brown), NF-κB (brown), and α-SMA (red). Bars = 100 μm. (**B**) Graphs showing quantitative data shown as box plots. (**a**) Cell number of α-SMA-positive cells, counted within the intima along the 100-μm circumferential length (left), and cell density over the 0.01 mm^2-intimal area (right). (**b**) relative number of α-SMA-VCAM-1 double-positive cells against the all α-SMA positive cells, (**c**) relative number of NF-κB-positive cells against the total number of cells. "C" and "V" indicate control group and vasculitis one at day 90, respectively. The number of animals used for the quantification is indicated in parentheses.

arteries at day 7 (Fig. 3B-c). The NF-κB(+)/VCAM-1(+) cells could still be identified in the intima and media at day 90. In contrast, neither VCAM-1(+)/α-SMA(+) nor NF-κB(+) cells were detected in control rabbits (left panels in Fig. 3A). Taken together, these results indicate that activation of SMCs is sustained in the coronary artery long after an episodic affectation of KD, likely contributing to aberrant remodeling of post-inflamed coronary arteries.

IV. Discussion

In the present study, we demonstrated post-inflammatory intimal thickening of coronary arteries at the chronic phase of the KD-like vasculitis. We found that inflammatory cells were barely detected in the coronary artery, but instead, observed were proliferation of vascular SMCs and excess accumulation of proteoglycans in the thickened lesion [21, 25], similar to the histological find-

ings reported from human autopsies [11, 37].

The thickened intima devoid of the inflammatory cells is quite distinct from the classical atherosclerotic intima, where inflammatory cells contribute to the development of atherosclerotic lesion according to "*the response-to-injury hypothesis*" [35]. Our observations indicate that α-SMA-positive cells and proteoglycans would contribute to the persistent pro-inflammatory signals of those lesions [2]. We assume that the sustained activation of vascular SMCs plays a pivotal role in unfavorable tissue remodeling in post-inflamed coronary arteries through excess production and secretion of proteoglycans [14, 42]. Although we have no precise identification of the constituents of proteoglycans, e.g., a core protein, versican [41], our assumption is in good agreement with a report on early histopathological changes in human coronary arteriosclerosis [27]. In the thickened lesion we observed no discernible accumulation of lipids even 60 days after development of KD-like vasculitis. This would be due to the feed of the rabbit; no high-fat diet was applied to augment the atherogenesis. Further study is required to address the development of authentic lipid-laden atherosclerotic lesions in our rabbit model.

We found accumulation of proteoglycans in the post-inflammatory thickening of the coronary artery, which are reportedly produced by activated SMCs or myofibroblasts [5, 8] via mediation of TGF-β signaling [19]. Of its various origins, TGF-β is secreted by regulatory T cells [17], which might be recruited by α-SMA-positive cells expressing VCAM-1 [36, 40]. Although we have not addressed the participation of T cells in our experimental vasculitis model, the elevated expression of VCAM-1 in SMCs observed in this study indicates that VCAM-1—T cell interaction could, at least in part, be involved in the increased production of proteoglycans in the lesion.

The increased proteoglycans are considered to be a double-edged deposit from the viewpoint of its pathophysiological consequence for vascular functions. They strengthen vascular stiffness and facilitate repair of the damaged tissue [1]. On the other hand, accumulation of proteoglycans could promote the progressive coronary artery stenosis, which may explain the sequela of the post-inflamed coronary artery after KD [33, 41]. Besides the thickening of the arterial wall, proteoglycans provide a scaffold for circulating oxidized low-density lipoproteins and monocytes to facilitate formation of atherosclerotic plaques [4, 14], which would augment the risk of IHD in the adolescent phase of post-KD patients.

The pathogenesis of post-KD remodeling discussed above suggests a possibility that the biochemical process for the production of proteoglycans might be a therapeutic target for prevention of the post-KD vascular remodeling. For example, an *in vitro* study indicated that glucosamine inhibits proteoglycan biosynthesis in vascular SMCs [20]. Targeting the TGF-β signaling pathway by, e.g., humanized monoclonal antibody (e.g. lerdelimumab) or synthetic inhibitors (e.g. LY-573636) for suppression of fibrosis or the tumor growth [6, 26], may be promising for post-KD remodeling. Suppression of VCAM-1 expression may also attenuate the remodeling. For example, expression of this adhesion molecule is reportedly attenuated by a synthetic HMG-CoA inhibitor, pitavastatin [44]. In practice, a clinical study indicates an anti-inflammatory effect of an HMG-CoA inhibitor fluvastatin for post KD adolescent patients [13]. Therapeutic efficacy of these agents would be worth investigating on the post-inflamed vascular remodeling after KD.

In summary, the present study provides a histopathological basis for pro-atherogenic lesion of the coronary artery long after the KD-like vasculitis. Our observations provide an important clinical implication; careful, continuous follow-up is required for post-KD young adults even with no obvious intimal thickening.

V. Disclosures

None.

VI. Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, 5461626 (C). We wish to thank Mr. Kawamura and Mr. Okuda for technical assistance in preparation of paraffin sections.

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