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Losartan Ameliorates Coronary Neointimal Thickening via Vascular Smooth Muscle Cell Phenotype Modulation in a Mouse Model of Kawasaki Disease

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Abstract

Background: Patients with Kawasaki disease (KD) who develop coronary artery aneurysms (CAAs) are at increased risk of future fatal coronary events. Pharmacotherapeutic strategies to prevent coronary stenosis are still lacking. This study investigated the therapeutic effect of the angiotensin receptor blocker (ARB) losartan, on coronary artery stenosis in a murine model. **Methods:** Five-week-old male C57BL/6J mice were intraperitoneally injected with 1,000 μg of *Lactobacillus casei* cell wall extract (LCWE) (n=12) to induce coronary artery stenosis. Two weeks later, LCWE-injected mice (n=12) were divided into two groups: six received drinking water containing losartan (100 mg/L) (LCWE+ARB), while six received normal drinking water (LCWE group). A control group (n=5) received phosphate-buffered saline (PBS) instead of LCWE. Sixteen weeks after LCWE administration—corresponding to the peak of coronary artery stenosis and 14 weeks after treatment initiation—the mice were euthanized for histological evaluation of the coronary arteries. **Results:** Losartan treatment significantly reduced the coronary arteritis score (4.3±3.3 vs. 19.3±2.8, p=0.003). LCWE-induced neointimal formation with vascular smooth muscle cell (VSMC) proliferation and subsequent coronary artery stenosis were markedly attenuated in losartan-treated mice (25% vs. 100%, p<0.001). Moreover, losartan inhibited coronary artery stenosis, at least in part, by preventing the phenotypic switch of vascular VSMCs from a contractile to a synthetic phenotype. **Conclusions:** Losartan is a potential therapeutic agent for preventing coronary events in KD by suppressing intimal proliferation and modulating the VSMC phenotype.

Keywords: angiotensin receptor blocker; *Lactobacillus casei* cell wall extract; neointima; coronary stenosis; vascular smooth muscle cell; elastin degradation

1. Introduction

Kawasaki disease (KD) is an acute febrile illness of unknown etiology that primarily affects infants and young children; it was first described by Tomisaku Kawasaki in 1967 [1]. The most serious complication is a coronary artery aneurysm (CAA). According to the latest nationwide epidemiological survey of KD in Japan, the incidence of CAA has decreased to approximately 2–3% with the availability of multiple therapeutic options, including intravenous immunoglobulin (IVIG) [2], corticosteroids such as prednisolone (PSL) [3], infliximab [4], cyclosporine A [5], and plasma exchange therapy [6]. However, children with CAA remain at increased risk for serious coronary events, particularly thrombosis, coronary stenosis, myocardial infarction, and even sudden death [7]. Autopsy studies have reported various vascular wall changes associated with CAA, including

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intimal thickening, medial destruction, and calcification [8]. To improve the prognosis for these patients, drugs that suppress intimal hyperplasia are urgently needed.

Angiotensin receptor blockers (ARBs) are widely used to prevent cardiovascular events such as myocardial infarction in patients with hypertension, heart failure, and atherosclerosis [9–11]. Basic research has demonstrated that ARBs have a wide range of beneficial effects beyond blood pressure reduction. These include the preservation of vascular endothelial function [12], anti-inflammatory [13] and antioxidant effects [14], and the inhibition of vascular smooth muscle cell proliferation [15]. CAAs associated with KD are also thought to share many similarities with cardiovascular diseases, including atherosclerosis and aneurysms, in that the structure of the vessel wall is destroyed by panvasculitis. In KD, CAAs are associated with an increased long-term risk of fatal or nonfatal coronary events [16]. Beyond aneurysm formation itself, progressive localized stenosis has been observed during follow-up, often at the inlet or outlet of aneurysmal segments [17,18]. These findings suggest that active vascular remodeling, rather than a simple aneurysm persists alone, underlies the development of coronary obstruction. This supports further investigation into the mechanisms of neointimal formation and stenosis in KD-related coronary vasculopathy.

In our previous basic research, we demonstrated that the combination of losartan, an angiotensin receptor blocker, and IVIG significantly inhibited coronary perivasculitis and myocarditis in mice injected with *Lactobacillus casei* cell wall extract (LCWE) [13]. Furthermore, we recently reported that the clinical use of ARBs or ACE inhibitors in combination therapy led to the regression of medium- or large-sized CAAs in patients with KD in long-term observation [19]. We have also established an animal model in which intraperitoneal injection of LCWE induced extensive coronary arteritis, followed by progressive coronary artery stenosis due to intimal thickening [20]. Based on these findings, the present study investigated whether ARB therapy attenuates vasculitis-related coronary artery stenosis in a murine model of KD, with a particular focus on the modulation of vascular smooth muscle cell (VSMC) phenotype and neointimal formation.

2. Materials and Methods

2.1. Animals

Five-week-old male C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan) and acclimated for 1 week prior to experimentation. The mice were housed under specific pathogen-free conditions in groups of 3 per cage and maintained on a 12h light/dark cycle at an ambient temperature of 23–25 °C and a relative humidity of 45–55%, with ad libitum access to standard rodent chow and water. All the experimental procedures were conducted in accordance with the institutional guidelines and regulations of Saitama Children's Medical Center, Japan. The study protocol was approved by the Animal Experimental Ethics Committee of Saitama Children's Medical Center (approval numbers: 2020-003 and 2021-003). All experiments were performed in compliance with the ARRIVE guidelines (ARRIVE guidelines 2.0).

2.2. Preparation for LCWE

LCWE was prepared as described previously [20,21]. Briefly, *Lactobacillus casei* (ATCC 11578; American Type Culture Collection, Manassas, VA, USA) was cultured in MRS broth (BD Difco, Franklin Lakes, NJ, USA) at 37° C for 48 h. The bacterial cultures were subsequently washed several times with phosphate-buffered saline (PBS), after which the pellet was resuspended (5 g wet weight in 15 ml of PBS). The suspension was sonicated for 2 h in a cooling dry ice/ethanol bath using a Q500 sonicator with a 3/4-inch probe at an amplitude ranging from 70 to 80% (Q Sonica LLC, CT, USA). The lysate was centrifuged at 20,000 × g for 1 h at 4 °C, and the supernatant containing the cell wall extract was collected. The concentration of LCWE was determined based on the rhamnose content using a phenol–sulfuric acid colorimetric assay and adjusted to 5 mg/ml in PBS. For disease induction, the mice received an intraperitoneal injection of 1,000 μg of LCWE (0.2 mL).

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2.3. Experimental Protocol

After acclimation, the mice were randomly assigned to experimental groups using a simple randomization method. A total of 12 mice received an intraperitoneal injection of LCWE (1,000 μg), and 2 weeks later, they were allocated into two groups (n = 6 per group):

- LCWE group: received normal drinking water
- In the LCWE+ARB group, the mice received drinking water containing losartan (100 mg/L; Sigma–Aldrich Co. LLC, St. Louis, MO, USA) for 14 weeks.

The control group (n = 5) received PBS instead of LCWE and was provided with normal drinking water on the same schedule. The water bottles were replaced weekly. Investigators performing histological and immunohistochemical analyses were blinded to group allocation. No animals were excluded from the analysis. At the end of the experimental period, the mice were euthanized under isoflurane anesthesia. Blood samples were collected via cardiac puncture, and hearts were harvested for histological analysis. Humane endpoints, including significant weight loss (<20%), reduced mobility, or signs of distress, were monitored throughout the study; however, no animals reached these endpoints. The dose of losartan was selected based on our previous study demonstrating its inhibitory effect on LCWE-induced coronary inflammation [13].

2.4. Histological Evaluations

As previously described, the coronary artery (CA) inflammation score was graded using a four-point scoring system: 0, no inflammatory cell infiltration; 1, inflammatory cells localized to the adventitia; 2, inflammatory cells extending into the intima and adventitia; and 3, inflammation involving all layers of the arterial wall (panvasculitis) [20]. The total inflammation score was calculated from 10 sections per animal, including five sections from each CA.

CA stenosis was evaluated using three parameters: the incidence of neointima (%), intimal thickness (μm), and the percentage of luminal stenosis (%). The incidence of neointima was defined as the proportion of animals exhibiting intimal thickening of the CA within each group. Intimal thickness and luminal stenosis were quantified using the following formula:

$$CA\ stenosis\ (\%) = \frac{Area\ within\ internal\ elastic\ lamina\ (IEL) - Luminal\ area)}{Area\ within\ IEL} \times 100$$

Elastin disruption was assessed using a semi-quantitative scoring system: 0, no disruption of the elastic lamina; 1, fewer than 10 disruptions of the elastic lamina; 2, more than 10 disruptions of the elastic lamina; and 3, marked weakening or disappearance of the elastic lamina. Elastin disruption was defined as the interruption of the elastic lamina followed by the reappearance of the laminar structure. The number of interruptions was counted across five consecutive sections. Both the internal elastic lamina (IEL) and the external elastic lumina (EEL) of the bilateral CA were evaluated. Morphometric measurements were performed using NIS-Elements AR software (version 5.11.00; Nikon Instruments Inc., Tokyo, Japan).

2.5. Immunohistochemistry

Formalin-fixed, paraffin-embedded cardiac tissue sections (2.5 μm-thick) obtained from experimental mice were immunostained using the following primary antibodies: anti-α-SMA (1:1000, clone 1A4, DAKO), anti-PCNA (1:800, ab18197, Abcam), anti-calponin (1:1000, ab46794, Abcam), and anti-MMP-9 (1:400, c-21733, Santa Cruz Biotechnology). For each staining procedure, isotype control sections processed without the primary antibody were included and showed no specific staining. Quantification of α-SMA expression was performed by counting positively stained cells in both the intima and media. PCNA expression was quantified in the intima only, whereas calponin expression was assessed in the media only.

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2.6. Measurement of Serum Cytokines by ELISA

Serum samples were obtained from mice by direct cardiac puncture under isoflurane anesthesia at the time of euthanasia. The collected blood was centrifuged to obtain serum, and the samples were stored at −80 °C until analysis. Serum levels of matrix metalloproteinase-9 (MMP-9), tissue inhibitor of metalloproteinases-1 (TIMP-1), platelet-derived growth factor-AA (PDGF-AA), and transforming growth factor-β (TGF-β) were measured using multiplex immunoassays. MMP-9 and PDGF-AA levels were quantified using the Luminex Mouse Discovery Assay (Cat. #F-RD-LuminexMM-02). TGF-β was measured using the MILLIPLEX Multi-Species TGF-β1 Panel Single-Plex (Cat. #F-MIL-TGFBMAG-64K-01). TIMP-1 expression was measured using the Luminex Mouse Discovery Assay (1-Plex) (Cat. #F-RD-LuminexMM-01). All measurements were performed by an external laboratory (Filgen Inc., Aichi, Japan) in accordance with the manufacturer's instructions. The concentrations of all the analytes are expressed in pg/mL.

2.7. Statistical Analysis

Continuous variables are expressed as the mean ± standard deviation (SD), and categorical variables are expressed as numbers and percentages (%). Differences among the three groups were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc correction. Categorical variables were compared using the chi-square test or Fisher's exact test, as appropriate. All statistical tests were two-tailed, and $p < 0.05$ was considered statistically significant. Statistical analyses were performed using SPSS version 24.0 (IBM Corp., NY, USA).

3. Results

The effect of losartan on LCWE-induced coronary arteritis is shown in Figure 1. In LCWE-treated mice, the incidence of coronary arteritis was 100%, whereas losartan treatment reduced the incidence to one third ($p < 0.01$ vs. LCWE). Furthermore, the coronary artery inflammatory score was significantly higher in LCWE-treated mice than in PBS-treated mice (0.0 ± 0.0 vs. 19.3 ± 2.8, $p = 0.001$), whereas losartan treatment resulted in a significant reduction in the score (4.3 ± 3.3, $p < 0.003$ vs. LCWE group).

Our previous study demonstrated that intimal thickening in LCWE-treated mice progresses over time [19]. In the present study, we investigated the effect of losartan on neointima formation. LCWE-injected mice developed marked intimal thickening rich in α-SMA–positive cells; however, this change was significantly attenuated by losartan treatment, resulting in improved coronary stenosis (Figure 2A–D). αSMA staining revealed that LCWE injection led to a loss of αSMA-positive cells in the media, accompanied by an accumulation of αSMA-positive cells in the neointima. Losartan treatment restored the predominant localization of αSMA-positive cells to the media (Figure 2A, 2E). The number of calponin-positive medial cells, a well-established marker of contractile/quiescent VSMCs, was significantly reduced in LCWE-treated mice but was increased in losartan-treated group (Figure 2F). Furthermore, the number of intimal PCNA-positive cells, a marker of vascular smooth muscle cell proliferation, was markedly increased following LCWE injection but was significantly suppressed by losartan treatment (Figure 2G).

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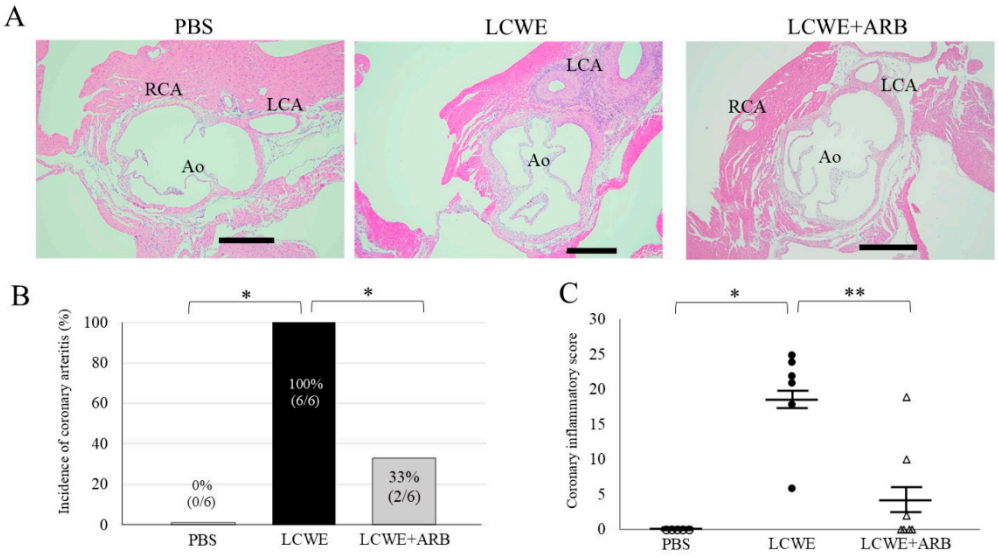

Figure 1. Anti-inflammatory effect of losartan on LCWE-induced coronary arteritis. Representative images show hematoxylin and eosin (H&E) staining of aortic root cross sections including bilateral coronary arteries from PBS-injected (left) or LCWE-injected mice (middle) and LCWE-injected mice treated with ABR (right). Bar graphs show the incidence of coronary arteritis (b) and the results of quantitative analysis of coronary inflammation (c) in mice treated with PBS, LCWE alone, and mice treated with LCWE and ARB. Scale bar indicates 500μm. *p<0.05. LCWE, *Lactobacillus casei* cell wall extract; ARB, angiotensin receptor blocker; RCA, right coronary artery; LCA, left coronary artery; Ao, aorta.

Elastica van Gieson staining revealed disruption of the vascular wall architecture in LCWE-injected mice, characterized by fragmentation of both the internal and external elastic laminae (Figure 3A). The elastin break scores of the IEL and EEL were significantly elevated in LCWE-injected mice than in control mice (IEL: 0.8 ± 0.4 vs. 21.2 ± 1.7, $p < 0.001$; EEL: 0.2 ± 0.2 vs. 28 ± 3.4, $p < 0.001$). In contrast, compared with the LCWE alone, the losartan treatment significantly ameliorated elastin disruption, with IEL and EEL scores reduced to 5.0 ± 2.8 and 6.3 ± 4.2, respectively ($p < 0.001$ for both). Serum MMP-9 levels were significantly reduced in the losartan-treated group than in the LCWE group ($p = 0.029$; Figure 4A). Furthermore, the expression of TIMP-1, an endogenous inhibitor of MMP-9, tended to decrease in the losartan-treated group (Figure 4B). Notably, the MMP-9/TIMP-1 ratio was not significantly different between the groups (data not shown). In contrast, no significant differences were observed in serum TGF-β or PDGF-AA levels among the three groups. (Figure 4C, D).

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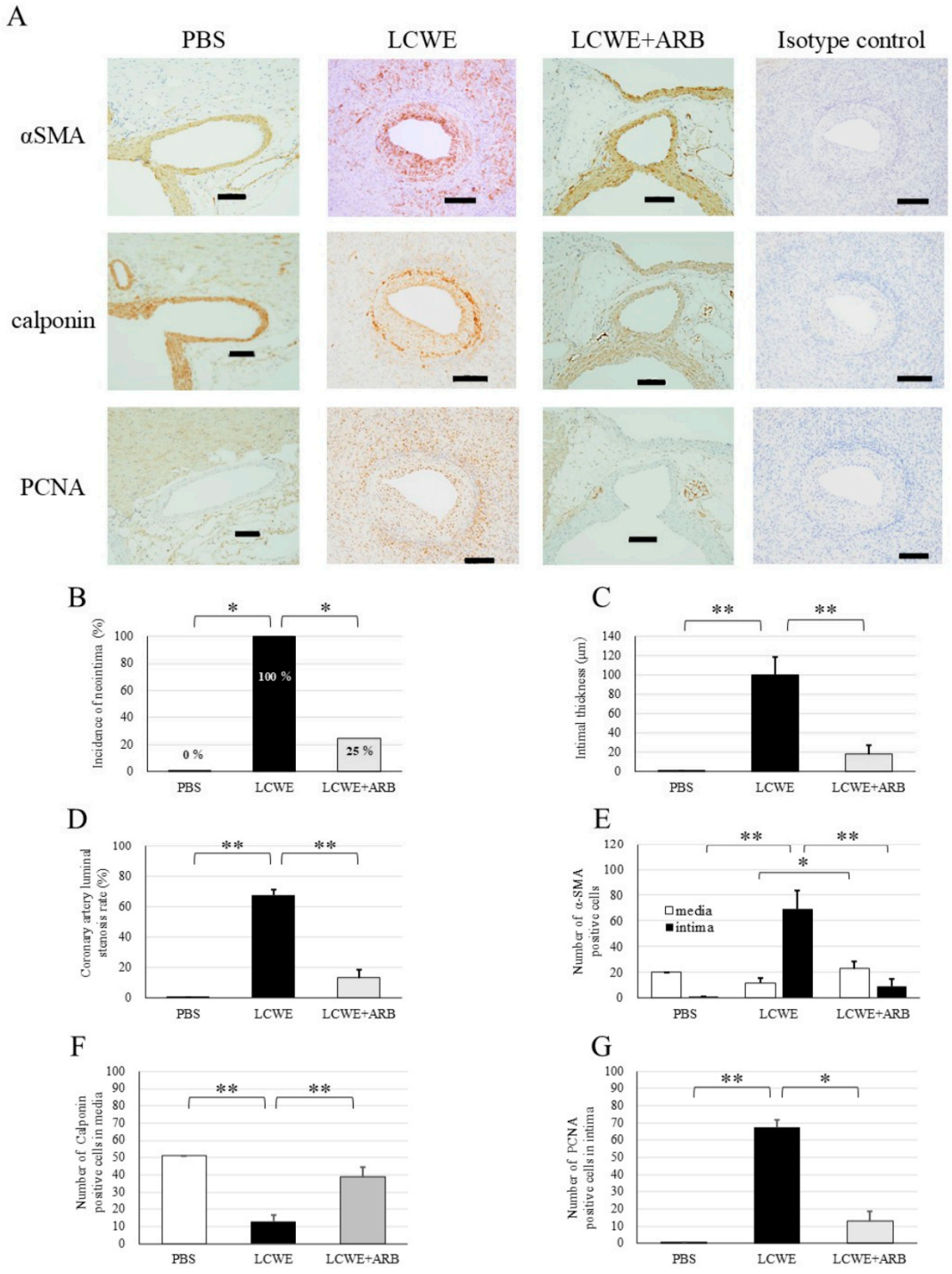

Figure 2. Immunohistochemistry of coronary artery stenosis. (A) Representative figures show H&E, α-smooth muscle actin (αSMA), calponin, and PCNA staining of coronary artery cross sections in upper cardiac sections isolated from 5-week-old C57BL6J mice that were intraperitoneally administered with PBS (PBS group) or LCWE followed by no treatment (LCWE group) or ARB treatment (LCWE+ARB). Scale bar indicates 100μm. The incidence of neointima (B), intimal thickness (C), coronary artery luminal stenosis rate, the number of αSMA-positive cells (E), calponin-positive cells (F), and PCNA-positive cells (G) were compared among the three groups. Date is expressed as the mean±SD. *p<0.05, **p<0.01. PBS, Phosphate-buffered saline； LCWE, *Lactobacillus casei* cell wall extract； ARB, angiotensin receptor blocker; H&E, hematoxylin and eosin; αSMA, α-smooth muscle actin; PCNA, proliferating cell nuclear antigen.

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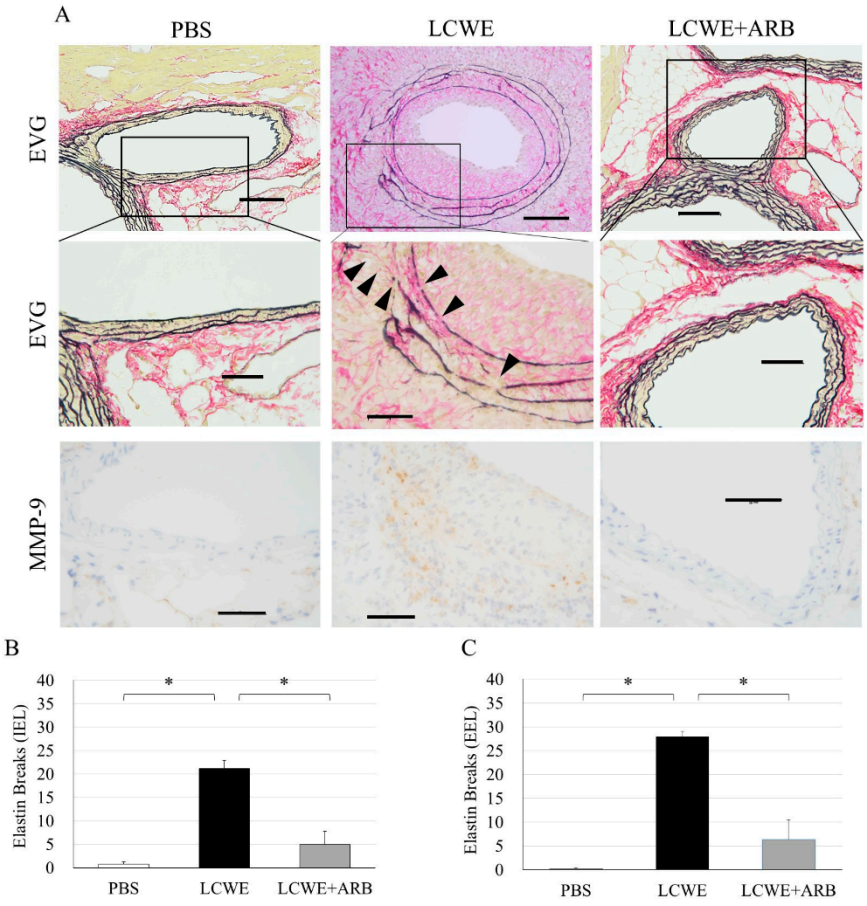

Figure 3. Effect of losartan on LCWE-induced elastin degradation. (A) Representative Elastica van Gieson (EVG)–stained cardiac tissue sections from mice treated with PBS, LCWE, or LCWE+ARB. Enlarged views of boxed regions are shown in the middle panels. Arrowheads indicate site of elastic fiber fragmentation in LCWE-injected mice. The lower panels show high-magnification images of MMP-9 staining. In LCWE-treated mice, increased MMP-9 expression (brown staining) was observed in regions corresponding to sites of elastic fiber fragmentation. Quantification of elastin breaks in the external elastic lamina (EEL) (B) and internal elastic lamina (IEL) (C) in each group (PBS: n = 5; LCWE: n = 6; LCWE+ARB: n = 6). Scale bars: 100 μm (upper panels) and 20 μm (middle and lower panels). *p<0.001.

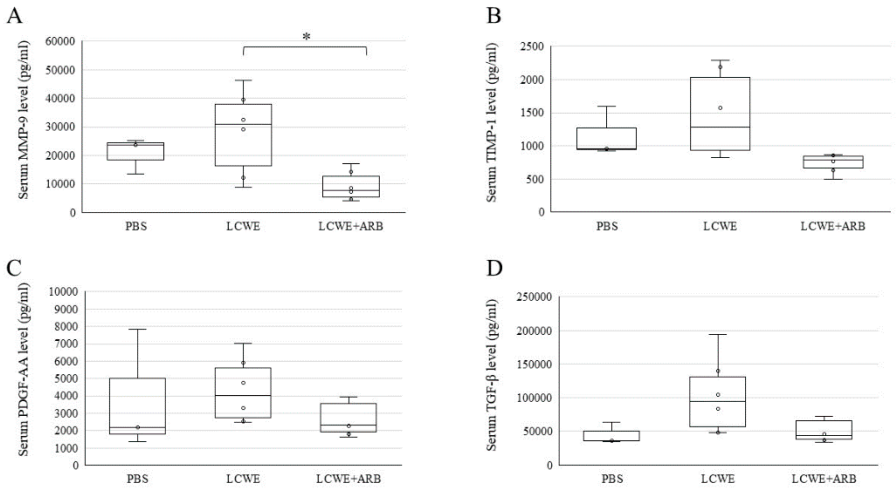

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Figure 4. Serum concentrations of MMP-9 (A), TIMP-1 (B), PDGF-AA (C), and TGF-β (D). Data are expressed as median (interquartile range [IQR]). MMP-9, matrix metalloproteinase-9; TIMP-1, tissue inhibitor of metalloproteinase-1; PDGF-AA, platelet-derived growth factor-AA; TGF-β, transforming growth factor-β. *p = 0.029.

4. Discussion

First, an important finding of the present study is that losartan treatment significantly suppressed coronary arteritis in the LCWE-induced murine model. Our findings further support the anti-inflammatory effects of losartan in coronary vasculitis. In our previous study using the same model, losartan significantly attenuated acute coronary perivasculitis and myocarditis and suppressed local and systemic inflammatory responses [13]. This finding is consistent with prior experimental evidence showing that suppression of key inflammatory pathways, including TNF-α and IL-1β signaling, attenuated coronary arteritis in KD mouse models [22,23]. Notably, TNF-α appears to play an important role in acute cardiac inflammation, whereas IL-1β contributes to subsequent coronary vasculitis, this suggests that therapeutic interventions that limit ongoing vascular inflammation may be beneficial even after inflammation has already been initiated [24].

Next, in the present study, losartan markedly attenuated LCWE-induced coronary artery stenosis. These findings are consistent with those of previous experimental studies showing that angiotensin II (AII) type 1 receptor blockade suppresses vascular restenosis and neointimal hyperplasia after balloon injury. Kauffman et al. demonstrated that losartan dose-dependently reduced neointimal thickening after balloon injury in rat carotid arteries, supporting a role for AII–AT1 signaling in injury-induced vascular remodeling [25]. In addition, Moon et al. reported that local delivery of losartan effectively prevented recurrent stenosis after balloon angioplasty, partly through inhibition of smooth muscle cell cycle progression and migration [26]. In our study, most cells within the neointimal region were αSMA-positive, suggesting a dominant contribution of VSMCs to coronary stenosis in this model. Therefore, we investigated the regulatory mechanism of losartan on the VSMC phenotype during neointimal formation.

In the present study, losartan markedly reduced the number of intimal PCNA-positive cells and was associated with the preservation of calponin-positive medial SMCs, suggesting the suppression of the synthetic/proliferative phenotype and maintenance of the contractile phenotype. Although direct evidence that losartan reverts VSMCs from a synthetic to a contractile phenotype remains limited, previous studies have shown that losartan suppresses AII–induced VSMC proliferation, migration, and inflammatory activation [27–29]. Losartan has been reported to inhibit cell-cycle progression and reduce proliferative activity in VSMCs [27], consistent with the marked reduction in the number of PCNA-positive cells observed in the present study. Moreover, recent studies have demonstrated that maintaining the differentiated/contractile phenotype of VSMCs is closely associated with the suppression of neointima formation [30,31]. Taken together, our results suggest that losartan may attenuate coronary artery stenosis by restraining the phenotypic activation of VSMCs and preserving a contractile medial phenotype, as supported by the retention of calponin-positive cells in the media.

In addition to its potential effects on VSMC phenotype, the inhibition of extracellular matrix degradation may contribute to losartan's anti-stenotic effect. MMP-9 has also been implicated in VSMC phenotype modulation, promoting the transition from a contractile state to a synthetic state [32]. Furthermore, it is involved in mechanisms that promote VSMC migration, phenotypic transition, and neointimal expansion by facilitating the degradation of elastic fibers [33–35]. Immunohistological findings from an autopsy case of KD revealed prominent MMP-9 expression in coronary lesions, indicating that MMP-9–mediated matrix degradation is involved in the pathogenesis of KD-related coronary artery abnormalities [36]. Therefore, the decrease in MMP-9 observed in this study suggests that preserving vascular wall structure and suppressing pathological remodeling may be beneficial in KD vasculopathy.

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This study has several limitations. First, the precise extent to which the anti-inflammatory effect of losartan contributes to the inhibition of coronary artery stenosis remains unclear. Coronary artery stenosis after the administration of LCWE progresses simultaneously with the progression of coronary arteritis [20]. However, in patients with KD, luminal myofibroblastic proliferation (LMP) characterized by unique SMC-derived pathologic myofibroblast, occurs in the subacute to chronic phase [37]. To isolate the direct effect on suppressing the progression of coronary artery stenosis, it is necessary to investigate the direct effect of losartan on the regulation of smooth muscle cell polarization in other experimental systems. Second, the antihypertensive effect of losartan treatment has not been thoroughly evaluated. Although the LCWE-induced murine model does not mimic the formation of coronary artery aneurysms, which are characteristic of KD patients. Furthermore, while we hypothesize that inhibiting intimal hyperplasia in this mouse model contributes to the reduction of diastolic blood pressure in response to vascular endothelial dysfunction [38] and destruction of vascular wall structure [39] caused by coronary arteritis. It is therefore imperative to examine the effects of calcium antagonists with antihypertensive properties on the mouse CA stenosis model.

5. Conclusions

In this study, losartan treatment attenuated coronary artery stenosis in an LCWE-induced murine model of Kawasaki disease. The protective effect of this ARB was associated with reduced neointimal formation and preservation of the contractile phenotype of VSMCs. These findings suggest that ARBs may inhibit the phenotypic switch of VSMCs from a contractile state to a synthetic state, thereby limiting intimal hyperplasia. The suppression of MMP-9 levels and the subsequent prevention of elastin degradation may further contribute to the maintenance of vascular wall integrity and the contractile VSMC phenotype. Taken together, these findings support the potential role of ARB therapy in preventing coronary events and improving outcomes in patients with CAAs associated with KD.

Author Contributions: E.S.: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Data curation, Writing—original draft. S.H., R.U., S.I., A.S., and M.T.: Investigation, Methodology. M.K.: Methodology, Supervision, Validation. A.N.: Conceptualization, supervision, writing—review and editing. All the authors have read and approved the final manuscript and agree to be accountable for all the aspects of the work.

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Institutional Review Board Statement: The animal study protocol was approved by the Institutional Review Board of the Animal Experimental Ethics Committee of Saitama Children's Medical Center (No: 2020-003, May 2021) for studies involving animals.

Informed: Consent Statement Not applicable.

Data: Availability Data is contained within the article or supplementary material.

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Conflicts of Interest: The authors declare no competing interests.

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