All-trans-Retinoic Acid Alleviates Coronary Stenosis by Regulating the Migration of Smooth Muscle Cells in a Mouse Model of Kawasaki Disease

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Research Article

Keywords: all-trans-retinoic acid, coronary artery stenosis, Lactobacillus casei cell wall extract

DOI: https://doi.org/10.21203/rs.3.rs-312272/v1

Abstract

Coronary artery (CA) stenosis is a detrimental and often life-threatening sequela in Kawasaki disease (KD) patients with coronary artery aneurysm (CAA). Therapeutic strategies for these patients have not yet been established. All-trans-retinoic acid (atRA) is a modulator of smooth muscle cell functions. The purpose of this study was to investigate the effect of atRA on CA stenosis in a mouse model of KD. Lactobacillus casei cell wall extract (LCWE) was intraperitoneally injected into 5-week-old male C57BL/6J mice to induce CA stenosis. Two weeks later, the mice were orally administered atRA (30 mg/kg) 5 days per week for 14 weeks (LCWE+atRA group, n=7). Mice in the untreated group (LCWE group, n=6) received corn oil alone. Control mice were injected with phosphate-buffered saline (PBS, n=5). Treatment with atRA significantly suppressed CA inflammation (19.3±2.8 vs 4.4±2.8, $p<0.0001$) and reduced the incidence of CA stenosis (100% vs 18.5%, $p<0.05$). In addition, atRA suppressed the migration of human coronary artery smooth muscle cells (HCASMCs) induced by platelet-derived growth factor subunit B homodimer (PDGF-BB). In conclusion, atRA dramatically alleviated CA stenosis by suppressing SMC migration. Therefore, it is expected to have clinical applications preventing CA stenosis in KD patients with CAA.

Introduction

Kawasaki disease (KD) is an acute systemic vasculitis of unknown etiology that is mainly associated with coronary artery aneurysms (CAAs) and occurs primarily in young children[1,2]. A serious complication of CAA is CA stenosis, which includes thrombotic occlusion of CA in the acute phase and progressive CA narrowing due to intimal hypertrophy in the chronic phase. These pathological features of CA stenosis were actually identified in autopsy cases[3]. Recently, Fukazawa R *et al.* performed a nationwide survey of KD patients with giant CAA in Japan. MI occurred in 18% of these patients, and severe cardiac events were likely to occur within 2 years of the onset of KD[4]. More recently, Miura *et al.* identified the risk factors for cardiac events in KD patients with CAA. Male sex and intravenous immunoglobulin (IVIG)-resistance were independent risk factors for acute coronary events[5]. Although these clinical studies revealed the natural history of KD patients with CAA, novel therapeutic strategies for these patients have not yet been investigated.

Recent clinical studies have reported useful therapeutic tools for anti-inflammatory agents such as IVIG[6], prednisolone[7], infliximab[8] and ciclosporin[9], especially during the acute phase. Therefore, according to the 25th Japanese nationwide Kawasaki disease survey, the incidence of cardiac sequelae was reduced to 2.6% in KD patients.[10] However, until now, due to the lack of a suitable animal model, no drug has been developed that is useful for improving the prognosis of KD patients who already have CAA. Pathological investigations of KD autopsy cases reported by Orenstein *et al* demonstrated that luminal myofibroblastic proliferation (LMP) was associated with unique smooth muscle cell (SMC)-derived myofibroblasts that also caused progressive CA stenosis[11]. Of note, we recently successfully discovered a novel mouse model of CA stenosis induced by *Lactobacillus casei* cell wall extract (LCWE) to mimic KD. Histologically, this model is characterized by intimal hypertrophy due to SMC proliferation and migration following

severe CA vasculitis, leading to CA stenosis[12]. Since the LCWE-induced vasculitis model closely resembled the pathological features found in human KD autopsy cases, we therefore decided to use this mouse as a model for chronic CA stenosis.

All-trans-retinoic acid (atRA) is a natural derivative of vitamin A that inhibits cell proliferation and migration and has anti-inflammatory properties[13-15]. Currently, atRA is a conventional therapy for the management of acute promyelocytic leukemia (APL)[16]. Recent experimental studies have reported that atRA treatment significantly reduces the formation of atherosclerosis in a high-fat diet-induced rabbit model[17]. Moreover, it has also been reported that atRA reduces neointimal formation in the carotid artery after balloon withdrawal injury in a rat model[18]. In addition, the synthetic retinoid Am80 significantly ameliorates *Candida albicans* water-soluble fraction (CAWS)-induced vasculitis through the inhibition of neutrophil migration and activation[19]. Based on previous animal studies, we hypothesized that atRA could suppress CA stenosis by modulating the properties of vascular smooth muscle cells (VSMCs) in a KD mouse model. In this study, we investigated the effect of atRA on LCWE-induced CA stenosis in a mouse model of KD.

Results

Preliminary evaluation of LCWE-induced CA stenosis

The mice were sacrificed 2, 4, 8 and 16 weeks after LCWE injection to investigate the natural history of LCWE-induced CA stenosis. Elastica van Gieson (EVG) staining revealed that CA intimal formation was first observed at 2 weeks and that the intimal thickness gradually increased over time. In addition to the increased intimal thickness, maximum vessel luminal narrowing was observed at 16 weeks after LCWE administration (Fig 1). Therefore, we chose to begin atRA treatment 2 weeks after LCWE injection and continued treatment for the next 14 weeks, which coincided with the onset of intimal formation and the time of maximum CA stenosis in this mouse model. None of the control mice that were injected with phosphate-buffered saline (PBS) exhibited pathological changes (data not shown).

Effect of atRA on CA inflammation and stenosis

Next, we evaluated the effects of atRA on CA inflammation. atRA (20 mg/kg) was orally administered 5 days per week from 2 to 16 weeks after LCWE injection. Inflammatory cells predominantly infiltrated the aortic root, and bilateral CAs were observed in LCWE-induced mice compared to PBS-treated mice. atRA significantly suppressed CA inflammation (19.3±2.8 vs 4.4±2.8, $p<0.0001$) (Fig. 2). Mice stimulated with LCWE exhibited CA stenosis in addition to vasculitis. We next assessed the effects of atRA on CA stenosis using three parameters. Representative microphotographs showing LCWE-induced CA intimal formation are shown in Fig. 3a. atRA significantly reduced intimal incidence (100% vs 18.5%, $p<0.05$), intimal thickness (100.5±18 vs 11.5±9.3 μm, $p<0.01$), and the CA stenosis rate (67.5 vs 7.6%, $p<0.01$) (Fig. 3b-d). α-Smooth muscle actin (αSMA)-positive cells in the thickened intima of the CA were predominantly observed in mice stimulated with LCWE. Proliferating cell nuclear antigen (PCNA)-positive and matrix

metalloproteinase (MMP)-9-positive cells were localized on the surface of the neointima but were not observed in mice treated with atRA (Fig. 4).

Effect of atRA on LCWE-induced elastin degradation through the suppression of MMP-9

Changes in the proliferative phenotype of SMCs precede elastolysis and are thought to play an important role in the development of intimal hyperplasia[20]. Therefore, assessing elastin degradation is extremely important for regulating intimal formation in the vessel wall. Next, we investigated the effect of atRA on the frequency of elastic breaks in the tunica media. LCWE-stimulated mice had more frequent interruptions and weakening of elastic fibers than mice that were administered PBS (Fig. 5a). atRA significantly reduced the elastin break scores of the external elastic lumina (EEL) (28±1 vs 6.9±3.4, $p<0.0001$) and internal elastic lumina (IEL) (21.2±1.7 vs 3.6±2.1, $p<0.0001$) (Fig. 5b,c). The potent electrolytic protein MMP-9, was increased in the serum of LCWE-induced mice (1.226±0.18 ng/ml) compared with PBS-injected mice (0.697±0.12 ng/ml, $p=0.66$). This LCWE-induced increase in MMP-9 was significantly suppressed in atRA-treated mice (0.674±0.12 ng/ml, $p=0.035$ vs LCWE group) (Fig. 5d).

Inhibitory effects of atRA on SMC migration in vitro

Next, human coronary artery smooth muscle cells (HCASMCs) were used to investigate the effect of atRA on HCASMC migration (Fig. 6a). The cell migration assay revealed that platelet-derived growth factor subunit B homodimer (PDGF-BB) stimulation increased the area covered by migrated cells (n=16, 515,703 μm^2) compared to that of medium alone (n=8, 443,594 μm^2, $p=0.04$). Cells were then treated with 0.1, 1.0, and 10 nM atRA for 72 h. The areas covered by migrated cells after treatment with 0.1 and 1 nM atRA were 407,610 (n=16) and 424,162 μm^2 (n=16), respectively. While these concentrations of atRA induced significant reductions of 21% ($p<0.0001$) and 18% ($p=0.002$), respectively, compared to those in the PDGF-BB-treated group, a greater reduction of 49% was observed in the 10 nM atRA treatment group (n=16, $p<0.0001$ vs. PDGF-BB, 0.1, 1 nM atRA treatment) (Fig. 6b).

Discussion

In the present study, we found that atRA dramatically reduced intimal hyperplasia and alleviated CA stenosis in an LCWE-induced model of KD vasculitis. CA stenosis is clinically caused by thrombus formation or intimal hyperplasia and induces cardiac events such as cardiac ischemia, MI, and even sudden death[2,4]. Vascular smooth muscle proliferation plays a pivotal role in the development of intimal hypertrophy, causing CA stenosis in KD patients with CAA, as evidenced by autopsy studies[9]. Therefore, this is the first report focused on the prevention of CA stenosis by regulating the properties of SMCs in a mouse CA arteritis model.

atRA is the most active metabolite of vitamin A. Numerous studies have reported that atRA has biological effects on various types of tumors, including breast and lung cancer and APL[21]. In recent years, atRA has been used as the standard therapeutic drug for the treatment of adult APL and pediatric neuroblastoma[22]. On the other hand, several basic experimental studies of cardiovascular disorders have

shown that atRA has antiproliferative and antimigratory effects in animal models of intimal hyperplasia. Miano *et al.* showed that atRA reduced neointimal formation and promoted favorable geometric remodeling of the rat carotid artery after balloon withdrawal injury[18]. In addition, Zhang *et al.* showed that atRA suppressed neointimal hyperplasia and inhibited VSMC proliferation and migration through direct activation of AMP-activated protein kinase (AMPK) and inhibition of mTOR signaling[23]. Therefore, we hypothesized that atRA might exert beneficial effects on the CA stenosis mouse model we developed in recent years. These previous data indicated that atRA improved intimal proliferation mainly associated with αSMA-positive cells. However, the effectiveness of atRA on cardiovascular disorders in clinical practice has not yet been verified. In addition, several clinical studies have investigated the relationship between retinol binding protein 4 (RBP4) and KD. Kimura *et al.* showed that RBP4, which is a candidate diagnostic marker, was decreased in patients with acute KD[24]. Recently, Yang *et al.* reported that KD patients had significantly lower RBP4 levels than healthy controls, suggesting that RBP4, which is a main retinol transport protein, is closely associated with markers of inflammation and thrombogenesis in children with KD[25,26].

Notably, compared to untreated mice, mice treated with atRA had significantly reduced CA inflammatory scores. This anti-inflammatory effect was consistent with the data reported by Miyabe *et al.* Am80, a retinoic acid receptor (RAR) agonist, has been shown to ameliorate mouse vasculitis induced by CAWS by suppressing neutrophil migration and activation[19]. In our study, the underlying pathophysiological mechanism of the anti-inflammatory effect of atRA remains unclear, but it is hypothesized that the SMC phenotype predisposes patients to increased proliferation and migration and contributes to persistent inflammation of the vessel wall.

We found that atRA significantly decreased elastin breaks and suppressed serum MMP-9 activity. A previous study by Axel *et al.* revealed that atRA inhibited human SMC proliferation and significantly inhibited the protein expression and activity of MMP-2 and MMP-9 in vitro[27]. More recently, Xiao *et al.* reported that atRA attenuated the progression of angiotensin II-induced abdominal aortic aneurysms by downregulating MMP-2 and MMP-9 expression in abdominal aortic tissue in apolipoprotein E-knockout mice[28]. In addition, Bunton *et al.* reported that phenotypic alterations in VSMCs preceded elastolysis in a mouse model of Marfan syndrome[20]. Therefore, it is reasonable to hypothesized that atRA protects against elastin degradation through the downregulation of MMP-9 activity, which in turn results in the suppression of proliferative phenotypic switching and the inhibition of intimal hyperplasia.

In vitro, we showed direct inhibitory effects of atRA on migration using HCASMCs stimulated with PDGF-BB. Several in vivo and in vitro studies have investigated the pathways that regulate the migration or proliferation of VSMCs. Day *et al.* first reported that atRA inhibited airway SMC migration by modulating the phosphatidylinositol 3 kinase (PI3K)/Akt pathway[29]. In addition, Zhang *et al.* demonstrated that atRA might inhibit neointimal hyperplasia and suppress VSMC proliferation and migration by direct activation of AMP-activated protein kinase (AMPK). They concluded that AMPK might be the pharmacological target of ATRA and that activation of AMPK by atRA may be a novel treatment strategy for

atherosclerosis[23]. More recently, Yu *et al.* reported that atRA prevented vein graft stenosis by inhibiting Rb-E2F-mediated cell cycle progression in human vein SMCs[30]. It was also reported that the Rb-E2F pathway was required for PDGF-BB-induced VSMC proliferation[31]. Our results confirmed that atRA inhibited PDGF-BB-induced HCASMC migration, suggesting an association between the Rb-E2F pathway and the antiproliferative effect of atRA.

Our research has some limitations. First, it was not possible to clearly determine whether atRA had a significant effect on inflammatory suppression or the induction of vascular repair. This is because we did not observe pathological features or changes in proinflammatory cytokines and chemokines over time. One possibility remains that atRA provides complete protection from the development of CA inflammation and stenosis during the experimental period. Treatment with atRA was started relatively early in this mouse model. Therefore, it was considered necessary to select a schedule for later administration of atRA treatment when CA inflammation had already developed. Second, the therapeutic goal of KD patients with CAA is to not only promote the regression of CA aneurysms but also prevent further cardiac events such as acute MI and sudden death. Thus, the beneficial effect of atRA on the suppression of intimal hyperplasia may lead to protection against CA stenosis and these harmful events. On the other hand, excessive inhibition of intimal hyperplasia may delay aneurysmal regression, resulting in a residual aneurysm. Therefore, it is important to induce favorable SMC proliferation rather than completely suppressing intimal hyperplasia leading to CA stenosis. Additional research is needed to elucidate the mechanism of the beneficial effects of atRA.

In conclusion, atRA dramatically reduced CA inflammation and stenosis by suppressing the production of MMP-9 and the migratory properties of SMCs by regulating cellular functions. Therefore, atRA, which has both anti-inflammatory effects and the ability to repair the vascular wall, is expected to have clinical applications to prevent CA stenosis in KD patients with CAA.

Methods

Experimental protocol

Five-week-old male C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan) and maintained in an environment with a 12 h light/12 h dark cycle under specific pathogen-free conditions. First, we examined the natural course of coronary stenosis in mice after LCWE administration. The mice were sacrificed on weeks 2, 4, 8, and 16 after intraperitoneal injection of 1,000 μg of LCWE (n=5 to 7 in each group). Cardiac tissue was harvested, and histological assessments were performed. Based on the pathological features of CA stenosis observed in the preliminary natural course experiment, the effects of atRA on CA stenosis in this mouse model of KD were examined. Five-week-old male C57BL/6J mice (n=13) were intraperitoneally injected with 1,000 μg of LCWE. Two weeks later, these mice were divided into two groups. Mice were orally administered atRA (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 20 mg/kg (n=7) or an equivalent volume (0.2 ml) of corn oil (n=6) using a disposable flexible-type gastric tube (Fuchigami, Kyoto, Japan) 5 days per week for 16 weeks. The same dose of atRA has been shown

previously to inhibit neointimal hyperplasia and suppress the proliferation and migration of VSMCs in mice[23]. Control mice (n=5) were injected with PBS instead of LCWE. All mice were killed at 18 weeks after LCWE or PBS administration. All mouse experimental procedures were performed in accordance with institutional guidelines and regulations of Saitama Children's Medical Center, Japan. This animal experiment was performed with the approval of the Animal Experimental Ethics Committee of Saitama Children's Medical Center (No: 2020-003). All experiments were performed in accordance with relevant ARRIVE guidelines.

LCWE preparation

LCWE was prepared as previously described[12,32]. Briefly, *Lactobacillus Paracasei subsp. paracasei* (ATCC 11578; American Type Culture Collection, Manassas, VA, USA) was cultured in MRS broth (BD Difco, Franklin Lakes, NJ, USA) for 48 h at 37 °C. The cells were harvested and washed with PBS, after which the cells were disrupted in 2 packed volumes of 4% sodium dodecyl sulfate (SDS) overnight at room temperature. Cell wall fragments were extensively washed 10 times with PBS to remove any residual SDS. The SDS-treated cell wall fragments were sonicated (5 g of packed wet weight in 15 ml of PBS) for 2 h using a Q500 sonicator with a 3/4" diameter probe at an amplitude setting of 70% (QSonica LLC, CT, USA). During sonication, the cell wall fragments were maintained by cooling in a dry ice/ethanol bath. The supernatant was centrifuged for 1 h at 20,000 g at 4 °C, and the supernatant containing the cell wall extract was used for the experiments. The concentration of LCWE was determined based on the rhamnose content as measured by a phenol-sulfuric acid colorimetric assay and adjusted to 5,000 μg/ml. To induce coronary arteritis/stenosis in mice, 1,000 μg (0.2 ml) of the LCWE preparation was injected intraperitoneally.

Histological evaluation

An inflammatory assessment of the CAs was performed as described previously[12]. Briefly, 2.5-μm sections of cardiac tissue were stained with hematoxylin and eosin (H&E) and EVG. Then, we selected 5 consecutive sections slightly distal to the bifurcation of the bilateral CAs. The intensity of coronary arteritis was scored with the following four criteria: 0, no inflammation; 1, inflammatory cells in only the adventitia; 2, inflammatory cells in both the intima and adventitia; and 3, panvasculitis as previously described[33]. The intensity of coronary inflammation was the total score of the bilateral CAs and is expressed as the inflammatory score of 10 CAs per individual animal. Elastin breaks were scored on the following scale: score 0, no interruption of elastic fibers; score 1, elastic breaks $\leq$10; 2, elastic breaks $>$10; and score 3, obscuration or disappearance of elastic fibers. Elastin breaks were defined as interruptions in the elastin fiber and the reappearance of the fiber and are expressed as the total number of breaks in five consecutive sections. The IEL and EEL of the bilateral CAs were evaluated. In addition, coronary stenosis was assessed according to three different parameters, including the frequency of neointimal formation, intimal thickness (μm), and coronary stenosis rate, which were measured with an NIS-Elements AR Ver5.11.00 (Nikon Instruments, Inc, Tokyo, Japan).

Immunohistochemistry

Paraffinized tissue sections (2.5 μm-thick) from experimental mice were stained with the following antibodies: anti-α-SMA (1:1000, SMA clone 1A4, DAKO), anti-PCNA (1:800, anti-PCNA antibody, Abcam) and anti-MMP-9 (1:400, MMP-9, Santa Cruz Biotechnology, Inc.). In each staining group, negative controls without the primary antibody were included and exhibited no staining.

Cell culture and migration assay

Primary HCASMCs were purchased from Thermo Fisher Scientific K.K. (Tokyo, Japan) These cells were cultured in Dulbecco's modified Eagle's medium (3.15 g/L glucose and 15 mM L-glutamine DMEM) (Lonza, Tokyo, Japan) containing 10% fetal bovine serum (FBS) and a penicillin-streptomycin-amphotericin B mixture (Lonza, Tokyo, Japan) at 37 °C in a humidified atmosphere containing 5% CO_2. The culture medium was changed every 24 h. Once the HCASMCs had grown to approximately 80 to 90% confluence, the cells were detached with a trypsin EDTA solution (Thermo Fisher Scientific, Tokyo, Japan). HCASMC migration assays were performed using an Oris cell migration assay kit (Platypus Technologies, LCC, WI, USA) according to the manufacturer's instructions. Briefly, cells were seeded at 10,000 cells/well in 96-well plates, and each well was coated with collagen I with a stopper in the central area to prevent the cells from adhering to the detection zone; the cells were incubated in serum-free medium for 24 h. Immediately after the stopper was removed, the cells were cultured with 20 ng/ml PDGF-BB in the absence or presence of various concentrations of atRA (0.1, 1.0, and 10 nM). After 72 h of incubation, cells that migrated from the perimeter to the detection zone were measured using an IN Cell Analyzer 2200 (Cytiba, MA, USA). The number of migrated cells in each well was counted in low-power (4×) fields.

Measurement of total MMP-9 activity

Blood samples were extracted directly from the left ventricle using a 1 ml syringe with a 27-gauge needle immediately before sacrifice under deep anesthesia. The blood samples were centrifuged at 1,500 rpm at room temperature and stored at -80 °C until use. The serum MMP-9 level was measured by an MMP-9 activity assay kit for mice (Cosmo Bio Co., Ltd, Tokyo, Japan) according to the manufacturer's instructions, and the data are expressed as ng/ml.

Statistical analysis

All values are presented as the mean ± standard error of the mean (SEM). Statistically significant differences between mean values were determined using a two-tailed Mann-Whitney test. Statistical differences among the three or more groups were determined by one-way ANOVA followed by Bonferroni *post hoc* test. Differences in which $p<0.05$ were considered statistically significant. IBM SPSS Statistics for Windows Version 24.0 (SPSS Japan, Tokyo, Japan) was used to analyze the data.

Declarations

Acknowledgments

We thank Mori Koki for preparing the LCWE. We would like to thank American Journal Experts (https://www.aje.com/) for English language editing.

Author contributions

E.S. designed and performed the animal experiments and wrote this manuscript. S.S. designed the experimental protocol and reviewed the manuscript. S.H. performed all steps of the histological procedures, including embedding, cutting, and staining. A.N. supervised the interpretation of the pathological findings and revised the entire manuscript. All authors have reviewed the manuscript.

Funding

This work was supported in part by JSPS KAKENHI grant number JP 19K08594 and a Grant-in-Aid Scientific Research (C), Kawano Masanori Memorial Public Interest Incorporated Foundation for Promotion of Pediatrics, and Saitama Children's Medical Center Research Grant.

Additional information

Competing interests

The authors declare no competing interests.

Data availability

All data generated or analyzed during this study are included in this published article.

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