Original article

Suppression of murine autoimmune myocarditis achieved with direct renin inhibition

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Background: The renin angiotensin system (RAS) plays an important role in the pathogenesis of cardiovascular diseases and inflammation. Myocarditis is an inflammatory disease of the heart, and the role of the RAS in its pathophysiology is unknown. Because the direct renin inhibitor, aliskiren, is thought to block RAS completely, we investigated the cardioprotective effect of aliskiren in mice with experimental autoimmune myocarditis (EAM).

Methods: A cardiac α-myosin heavy chain peptide was injected in mice on days 0 and 7. Aliskiren 25 mg/kg per day (n = 10) or vehicle (n = 10) was administered to EAM mice starting on day 0 and the animals were killed on day 21.

Results: Aliskiren significantly prevented the progression of left ventricular wall thickening in EAM hearts compared to the vehicle-treated group. Histologically, the inflammatory cell infiltration and fibrosis area ratios in the aliskiren-treated group were lower than that in the vehicle-treated group. Immunohistochemistry revealed that aliskiren suppressed CD4 positive cell infiltration in EAM hearts compared to vehicle. Moreover, aliskiren decreased mRNA levels of interleukin (IL)-2, interferon-γ, tumor necrosis factor-α, and collagen 1. In vitro study showed that aliskiren inhibited T cell proliferation and IL-2 production induced by myosin stimulation.

Conclusion: Our results suggest that aliskiren ameliorates EAM by suppressing T-cell activation and inflammatory cytokines, and has potential as a treatment for myocarditis.

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Introduction

Myocarditis is an inflammatory heart disease that poses many risks to humans. It is progressive, can cause severe heart failure, arrhythmia, shock, and death [1]. Some cases of myocarditis are related to autoimmunity and lead to chronic immune-mediated inflammation, resulting in dilated cardiomyopathy [2–4]. Experimental autoimmune myocarditis (EAM) induced by inoculation with cardiac myosin is a model of post-infectious myocarditis and cardiomyopathy [5–7]. EAM is mediated by CD4+ T cells [5,8].

The renin angiotensin system (RAS) plays an important role in the pathogenesis of atherosclerosis, hypertension, left ventricular hypertrophy, myocardial infarction, and heart failure [9,10]. In addition to systemic RAS, RAS local to various end organs can produce angiotensin II (Ang II) locally and cause tissue injury and affect tissue regeneration [11]. For example, cardiac RAS regulates cardiac hypertrophy, remodeling, and fibrosis [12–14]. In some studies, these changes were independent of blood pressure and plasma Ang II [13,14]. Local RAS is also known to promote inflammation. Ang II can modulate adaptive immunity, acting directly on lymphocytes. In addition to the classic components required for T cell activation, Nataraj et al. reported that Ang II stimulates the proliferation of splenic lymphocytes, including B
cells and T cells [15]. In addition, T cells are equipped with all RAS components such as the angiotensin I converting enzyme (ACE) [16], renin, and angiotensinogen. They are also potentially able to produce and deliver Ang II [17,18]. The effects of Ang II on T cells have been suggested to be mediated by Ang II type 1 receptors (AT1R) or Ang II type 2 receptors (AT2R). These conclusions are based not only on pharmacological inhibition with AT1R or AT2R blockers, but also on studies of T cells from AT1R- and AT2R-deficient mice [18,19]. However, the anti-inflammatory effect due to inhibition of other RAS components on T cells has not been fully demonstrated.

The action of renin on angiotensinogen is the first and rate-limiting step in the RAS cascade. Aliskiren is the first available direct renin inhibitor. It inhibits the catalytic activity of renin by binding to the active site of renin and reduces synthesis of all subsequent components of the RAS cascade. Previous studies reported that aliskiren treatment resulted in amelioration of cardiac hypertrophy and vascular inflammation, and improved systolic function, all beyond its blood pressure lowering effects in animal models [20–23]. These effects of aliskiren suggest that it blocks not only systemic RAS but also local RAS. RAS blockade by ACE inhibitors (ACEIs) and Ang II receptor blockers (ARBs) induce the activation of feedback mechanisms that result in increased renin and its downstream RAS components. Therefore, aliskiren may provide a more complete block of local RAS than ACEIs and ARBs [24]. However, the effect of aliskiren on local RAS and inflammation has not been fully elucidated.

In this study, we demonstrate for the first time that aliskiren suppresses acute myocarditis in EAM models through the inhibition of local RAS.

**Methods**

**Animals**

Male BALB/c mice (7 weeks old, 23–26 g) were purchased from Japan CREA Corporation (Tokyo, Japan). They were given a standard diet and water and were maintained in compliance with the animal welfare guidelines of the Institute of Experimental Animals of Tokyo Medical and Dental University. All animal experiments were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

**Inoculation (induction of experimental autoimmune myocarditis)**

A specific peptide derived from murine cardiac α-myosin heavy chain [Myhc-α, 614–620 (Ac-SLKLMLFSTYASAD-COOH)] (Japan Bio Services Corporation, Saitama, Japan) was used as an antigen as previously described [7,25]. The peptide was dissolved and emulsified with an equal volume of Freund’s complete adjuvant (DIFCO Laboratories, Detroit, MI, USA). Each mouse was injected subcutaneously in the back with 150 μg/200 μL of peptide on days 0 and 7.

**Aliskiren and administration**

Aliskiren was provided by Novartis Pharma AG (Basel, Switzerland). Aliskiren was dissolved in 0.5% carboxymethylcellulose (CMC) vehicle immediately before use. EAM mice received daily subcutaneous injections of aliskiren (25 mg/kg) (ALS25, n = 10), aliskiren (50 mg/kg) (ALS50, n = 6) from day 0 to day 21. The day 0 is the day that the mice received their first inoculation. We selected the dose based on a previous study conducted also in mice [26]. Drug-free vehicle (0.5% CMC solution) was administered to another set of EAM mice as a positive control (Vehicle, n = 10). Non-immunized mice hearts were also used as a negative control (Control, n = 5).

**Physiological examinations**

Blood pressure was measured in the conscious state on days 0 and 21 in all three groups of mice using a tail-cuff system (BP-98A, Softron Co., Tokyo, Japan). Transthoracic echocardiography was performed on days 0 and 21 with ultrasound equipment (Nemio, Toshiba, Tokyo, Japan) on animals anesthetized by intraperitoneal administration of 3.6% chloral hydrate. Hearts were imaged in the two-dimensional mode in short-axis views at the level of papillary muscle. Ejection fraction (EF) was calculated by the Teichholz method on M-mode. Total wall thickness was calculated as the sum of septum and left ventricular posterior wall thickness.

**Histological analysis**

All mice were killed under anesthesia by intraperitoneal administration of 3.6% chloral hydrate on day 21. The hearts were harvested immediately and the upper one-third of the ventricles was fixed in 10% formalin, embedded in paraffin, and sectioned. We obtained transverse sections for histological examination. The heart sections were stained by hematoxylin and eosin (HE) or Mallory method. The extent of inflammatory cell infiltration was measured using HE staining. The degree of fibrosis was calculated using Mallory staining. The area of the heart and region affected by myocarditis (consisting of inflammatory cell infiltration and fibrosis) was determined by a computer-assisted analyzer (Scion Image beta 4.02, Scion Corporation, Frederick, MD, USA). The area ratio (affected/entire area expressed as a percentage) was calculated as described previously [27].

**Immunohistochemistry**

The middle one-third of ventricles was embedded in O.C.T. compound (Sakura Finetek, Tokyo, Japan) tissue medium, frozen in liquid nitrogen and stored at –80 °C. Tissue blocks were sectioned at 6 μm. Sections were then incubated with anti-CD4 (SouthernBiotec, Birmingham, AL, USA), anti-CD8 (PharMingen, San Diego, CA, USA) or anti-renin (no. 593) antibody (each at 1–10 μg/mL) at 4 °C. Histofine simple stain was used as a secondary antibody. Incubations with secondary antibodies were carried out at room temperature for 30 min. After incubation with avidin-biotin-horseradish peroxidase complexes, counterstaining was performed with hematoxylin. Immunostained type- and class-matched nonimmune phosphate-buffered saline was used as the negative control for each antibody [27,28]. Measurement of immunoreactivity of myocardial infiltrating cells for CD4 and CD8 was performed in 25 randomly selected fields in heart sections at 400-fold magnification of light microscopy.

**Measurements of renin and angiotensin peptides**

Blood samples were taken from the abdominal aorta under anesthesia on day 21. Plasma renin activity (PRA) and plasma angiotensin I (Ang I) levels were measured by standard radioimmunoassay methods (SRL Co., Tokyo, Japan).

**Real-time polymerase chain reaction**

Total RNA was isolated from the EAM hearts on day 21 after immunization; native hearts were used for control. cDNA was prepared with a real-time polymerase chain reaction (RT-PCR) kit (Stratagene Co., La Jolla, CA, USA). RT-PCR in a StepOne real-time PCR system (Applied Biosystems, CA, USA) was used to determine the mRNA expression of interleukin (IL)-2 (Mm00434256_m1), interferon (IFN)-γ (Mm01168134_m1), IL-4 (Mm00445259_m1), and...
IL-10 (Mm00439616_m1), IL-17A (Mm00439618_m1), tumor necrosis factor (TNF)-α (Mm00443258_m1), collagen I (Mm01302043_g1), and 18s rRNA (Applied Biosystems) used as a control. The RT-PCR protocol consisted of an initial step at 95°C for 20 s followed by 50 cycles: 95°C for 1 s, and annealing at 60°C for 20 s. cDNA was run in duplicates and quantitative data were calculated using the comparative CT (ΔΔCT) method.

T cell proliferation assay and ELISA

Spleen cells were isolated from mice with myocarditis on day 21. Cells (5 × 10^5/well) were cultured in 96-well culture plates with 12.5 μg/ml MHC-α (64-634). Aliskiren was added to each well at different concentrations. Cultures were incubated at 37°C under CO2 for 72 h. T cell proliferation was assessed by MTT assay with Cell Counting Kit-8 (Dojindo, Tokyo, Japan). Cell proliferation was expressed using optical density. Concentrations of cytokines and Ang I in the culture supernatants were measured with enzyme-linked immunosorbent assay (ELISA) kits (Peninsula Laboratories, LLC, CA, USA) according to the manufacturer’s instructions.

Statistical analysis

Values are given as mean ± SEM. We used Student’s t-test to compare two groups. Multiple group comparison was analyzed by one-way ANOVA. The Bonferroni correction was used for post hoc analysis. Differences were considered statistically significant at a value of p < 0.05.

Results

Effect of aliskiren on blood pressure and PRA

The Vehicle mice had lower blood pressure than the Control mice (Fig. 1A). The blood pressure of ALS25 mice was not significantly lower than that of Vehicle mice (103 ± 2.7 mmHg in the Vehicle group vs 96.3 ± 3.0 mmHg in the ALS25 group, p = 0.27). However, that in the ALS50 group was significantly lower compared to the Vehicle mice (88.0 ± 4.4 mmHg in the ALS50 group, p = 0.02) (Fig. 1A). This result suggested that 25 mg/kg per day of aliskiren was a safe dose because it did not lower blood pressure. To investigate whether our dose of aliskiren affected circulating RAS components, we performed plasma biochemistry analyses. PRA and Ang I concentrations were significantly lower in the Vehicle than in the Control mice (Fig. 1B and C). In contrast, PRA and Ang I in the Aliskiren group were comparable to Control mice.

Aliskiren reduced heart weight to body weight ratios and left ventricular wall thickness

Heart and body weights were measured, and the ratio of heart to body weight (HW/BW) was calculated. Vehicle EAM hearts demonstrated increased HW/BW ratio (6.5 ± 0.3 mg/g) compared to that of native mice (4.9 ± 0.1, p < 0.05). Aliskiren administration significantly reduced this ratio (ALS25 5.4 ± 0.2, p < 0.05) compared to that of the Vehicle group (Fig. 2A). Left ventricular (LV) wall thickness and LVEF were analyzed by echocardiography on day 21 (Fig. 2B–E). Aliskiren significantly countered the progression of LV wall thickening of EAM hearts (2.37 ± 0.08 mm in Vehicle, 1.96 ± 0.05 mm in the ALS25, p < 0.05) (Fig. 2F). The LVEF was normal and comparable between the Vehicle- and aliskiren-treated groups (Fig. 2G). There was no significant difference between ALS25 group and ALS50 group in these parameters (Fig. 2A, F, G).

Aliskiren suppressed myocardial cell infiltration and fibrosis

Histological analysis was performed on day 21. The Vehicle mice showed severe myocarditis with inflammatory cell infiltration and destruction of myocardial fibers, but aliskiren attenuated this pathological change (Fig. 3A–D). The area of cellular
infiltration was significantly decreased in aliskiren-treated EAM mice (13.9 ± 2.5% in the Vehicle group vs 3.6 ± 0.7% in the ALS25 group, 3.8 ± 1.1% in the ALS50 group, p < 0.05) (Fig. 3I). Mallory staining also showed that fibrotic lesions were significantly fewer in the aliskiren-treated group compared to the Vehicle-treated group (17.6 ± 2.8% in Vehicle vs 7.3 ± 1.2% in ALS25, 6.8 ± 1.3% in ALS50, p < 0.05) (Fig. 3E-H, J). Control mice hearts showed no cell infiltration and fibrosis. There was no significant difference between ALS25 group and ALS50. To investigate the effect of aliskiren in EAM without lowering blood pressure, 25 mg/kg/day (ALS25) was used as the aliskiren group in the following study.

**Aliskiren suppressed CD4+ T cell infiltration**

Immunohistochemistry revealed that enhanced expression of CD4 on infiltrating cells was observed in Vehicle hearts, while aliskiren administration significantly suppressed this expression (Fig. 4A–C, C). The number of CD8-positive myocardial infiltration cells increased neither in the Vehicle hearts nor in the aliskiren hearts (Fig. 4D–F, H). There was no statistical difference.

**Effect of aliskiren on inflammatory cytokines**

RT-PCR analysis showed that the cardiac gene expressions of IL-2, IFN-γ, TNF-α, and collagen I were markedly higher in the Vehicle mice than in the control mice. In contrast, treatment with aliskiren significantly reduced the cardiac expression of these factors compared with those in Vehicle (n = 10) (Fig. 5A–D). On the other hand, there was no significant difference in the gene expression of Th2 cytokines, IL-4 and IL-10, and Th17 cytokine, IL-17, between the aliskiren and the Vehicle groups (Fig. 5E–G).

**Effect of aliskiren on local renin expression**

To investigate whether aliskiren inhibited local renin expression, we performed immunohistochemistry analysis. Renin was expressed on infiltrating cells in the EAM hearts. Aliskiren treatment attenuated this effect (Fig. 6). Native mouse hearts showed no enhanced expression of renin.

**Suppression of T cell proliferation**

To explore the direct effect of aliskiren on the proliferation of inflammatory cells, especially T cells, we stimulated spleen cells from EAM mice with cardiac myosin under different aliskiren concentrations. We found that T cell proliferation in response to cardiac myosin was suppressed by aliskiren in a concentration-dependent fashion (Fig. 7A). Moreover, ELISA analysis of supernatants after incubation of spleen cells with myosin revealed that production of IL-2 and Ang I was suppressed by aliskiren (Fig. 7B and C).
Discussion

In the present study, we showed that aliskiren attenuated myocardial cell infiltration and fibrosis in EAM mice. A low dose of aliskiren suppressed myocardial fibrosis and inflammatory cell infiltration without lowering blood pressure. We further demonstrated in vitro that aliskiren suppressed inflammatory cell proliferation.

Previous studies support the notion that there is a local RAS in the heart which locally produces Ang II [12–14]. It is thought that local cardiac RAS is independent of systemic RAS, because ACE inhibitors and AT1R blockers reduce cardiac hypertrophy or fibrosis without blood pressure or plasma Ang II concentration reduction [12–14]. Aliskiren, a direct renin inhibitor, is also reported to suppress local cardiac RAS. For instance, aliskiren improved LV dysfunction after myocardial infarction at a dose that did not affect blood pressure in an animal model [20]. In this study, we tried to verify the effects of aliskiren on cardiac injury and inflammation in EAM that was independent of blood pressure. Therefore, we used aliskiren at a low dose that was shown not to reduce blood pressure.

Our study showed that low-dose aliskiren was able to reduce cardiac fibrosis and cell infiltration in EAM without causing a blood pressure decline. Plasma Ang II concentration and PRA, which were measured on day 21, were higher in aliskiren-treated EAM mice than in Vehicle-treated EAM. This increase was attributed to a finding that the inhibitory effect of aliskiren on PRA tends to decrease over time [29]. Because of a counter-regulatory effect, chronic treatment of aliskiren causes increasing PRA and normalizing renal angiotensin levels. On the other hand, aliskiren eventually replaced tissue renin by aliskiren-bound renin and this effect lasted longer than in plasma. Then, the difference between the RAS activation in the tissue and in the plasma occurred [29]. The study showed that inhibition of systemic RAS did not fully explain the beneficial effects of aliskiren. Our findings on PRA and plasma Ang II likewise suggest an inhibitory effect of aliskiren on local RAS that is independent of its effect on systemic RAS.

Local RAS is known to be a potent proinflammatory mediator. Myosin-induced EAM is a CD4+ T cell, especially Th1-driven myocarditis. A previous study suggests that T cells are equipped with all RAS components and are potentially capable of producing and delivering Ang II to sites of inflammation [16–18]. We also showed that CD4+ T-cell infiltration and Th1 cytokine levels increased in untreated EAM mice. These changes were significantly ameliorated by aliskiren treatment, indicating that aliskiren suppressed proinflammatory cytokines and CD4+ T cell proliferation by inhibiting local RAS. Previous studies demonstrated that treatment with AT1R blocker downregulated Th1 cytokines and up-regulated Th2 cytokines in myocarditis rats [30]. In our study, the expression of IL-4 was slightly up-regulated, but IL-10 expression showed a tendency opposite to the previous studies. It is known that addition to Th2 cells, monocytes, and regulatory T cells also induces the production of IL-10. So, effectiveness of aliskiren on the IL-10 expression and the influence on the monocytes and regulatory T cells should be further analyzed in our models. Previous studies suggest that not only Th1 T cells, but also Th17 T cells play an important role in the progression of EAM [31,32]. Our data suggested that there was a trend toward decreased expression of IL-17A in aliskiren-treated EAM mice, but the difference was not statistically significant. Therefore, our data suggested that the effect of aliskiren was mainly related to the suppression of Th1 cytokines. In addition, the previous study

Fig. 3. Effect of aliskiren (ALS) on inflammatory cell infiltration and fibrosis in hearts of mice with experimental autoimmune myocarditis (EAM).

Representative slides with hematoxylin and eosin (HE) staining (A-D) and Mallory staining (E-H) are shown. Mice treated with aliskiren showed significantly less inflammatory cell infiltration (C and D) and fibrotic areas (G and H) compared to those in the vehicle-treated (B and F) EAM mice. Native mice hearts (A and E) showed no cell infiltration and fibrosis. Quantitative data of cell infiltration (I) and fibrosis (J) are demonstrated. Scale bars = 100 μm. *p < 0.05.
Fig. 4. Immunohistochemical staining of CD4 and CD8 on inflammatory cells.
Immunohistochemical analysis showing expression of CD4 (A–C) and CD8 (D–F) in the inflammatory cells of hearts from native and experimental autoimmune myocarditis (EAM) receiving vehicle only or receiving aliskiren. Arrows indicate CD8 positive cells. Quantitative data showing numbers of cells positive for CD4 (G) and CD8 (H) per mm². Scale bars = 100 μm.

Fig. 5. Cytokine and collagen mRNA expression in experimental autoimmune myocarditis (EAM) hearts. Quantitative data of mRNA levels of interleukin (IL)-2 (A), interferon (IFN-γ) (B), collagen I (C), tumor necrosis factor (TNF)-α (D), IL-4 (E), IL-10 (F), and IL-17 (G) are demonstrated. Group EAM, EAM mice administered with vehicle; group ALS, EAM mice treated with aliskiren. *p < 0.05 as compared to control EAM treated with vehicle.
reported that the chemokines monocytes chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α, acting through their receptors CCR2 and CCR5, were important in the induction of EAM [33,34]. In a different animal model of myocardial ischemia, it has been reported that aliskiren suppressed MCP-1 expression [35]. In our study, we did not examine the role of chemokines; therefore, further work is necessary to determine whether aliskiren might suppress the expression of chemokines that affect T cell migration in EAM mice.

Furthermore, aliskiren suppressed spleen cell proliferation and production of IL-2 and Ang I after incubation with myosin in vitro. Endogenous production of Ang II by T cells is important for T cell responses, such as activation and adhesion/transmigration [19,36]. Our results suggest that aliskiren reduced endogenously produced Ang II by inhibiting renin activity on T cells, which then blocked T cell activation and proliferation. In other animal models, it has been reported that aliskiren prevented cardiac inflammation, fibrosis, renal glomerulosclerosis, and cell infiltration [18,19]. Previous data and our observations support the notion that aliskiren has the potential to inhibit inflammation, which may be useful for treating and/or preventing myocarditis in clinical settings.

**Limitations**

We started aliskiren at the same time as the first myosin inoculation. Therefore, we do not know if aliskiren is able to reverse myocardial changes if it is started sometime after EAM formation, mimicking the clinical situation in which an infectious myocarditis, for example, is not diagnosed immediately.

In conclusion, we demonstrated for the first time, that the protection of myocardial cells from injury and inflammation in EAM mice by aliskiren was associated with depression of T cell proliferation together with the suppression of cytokine production. These results indicate that aliskiren may have potential as a
therapy for myocarditis. Further studies are needed to evaluate the effectiveness of this novel therapy in humans.

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Disclosures
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