Rivaroxaban Inhibits Angiotensin II-Induced Activation in Cultured Mouse Cardiac Fibroblasts Through the Modulation of NF-κB Pathway

Takehiro Hashikata, MD, Minako Yamaoka-Tojo, MD, Sayaka Namba, MD, Lisa Kitasato, MD, Ryo Kameda, MD, Masami Murakami, MD, Hiroe Niwano, MD, Takao Shimohama, MD, Taiki Tojo, MD, and Junya Ako, MD

SUMMARY

Cell migration, proliferation, and differentiation of cardiac fibroblasts (CFs) play a central role in cardiac fibrosis. Factor Xa (FXa)-dependent protease-activated receptor (PAR)-1 and PAR-2 have been reported as important targets in proinflammatory and fibroproliferative diseases. From this viewpoint, we aimed to investigate whether treatment of rivaroxaban, an approved oral direct FXa inhibitor, attenuates functional changes in angiotensin (Ang) II-induced mouse CFs.

Confluent cultured mouse CFs were pretreated with or without rivaroxaban. Ang II-induced cell migration was decreased by 73% in rivaroxaban induced cells. Rivaroxaban inhibited Ang II-induced cell proliferation by 27% at 0.01 μg/mL, 69% at 0.1 μg/mL, 71% at 1 μg/mL, and 69% at 5 μg/mL. In mouse cytokine array measuring 40 cytokines, the productions of interleukin-16, TIMP-1, and tumor necrosis factor-α (TNF-α) were significantly reduced with 0.1 μg/mL of rivaroxaban pretreatment (all P < 0.05). TIMP-1 levels in the culture supernatant measured by ELISA were also decreased by rivaroxaban pretreatment in Ang II-induced CFs (35% decrease at 0.01 μg/mL, 47% at 0.1 μg/mL, 47% at 1 μg/mL, and 57% at 5 μg/mL). In the dual reporter assay analysis, rivaroxaban inhibited various inflammatory signal pathways, including the nuclear factor-kappa B (NF-κB), active protein-1 (AP-1), and mitogen-activated protein kinase (MAPK) pathways (decreases of 82%, 78%, and 75%, respectively).

These data suggest that rivaroxaban inhibits Ang II-induced functional activation in cultured mouse CFs via inhibiting NF-κB and MAPK/AP-1 signaling pathways, which may be a possible target of heart failure, through the antifibrotic and anti-inflammatory efficacy of rivaroxaban in Ang II-stimulated cardiac fibroblasts. (Int Heart J 2015; 56: 544-550)

Key words: Factor Xa inhibitor, Cardiac fibrosis, Anticoagulant, Cell signaling, Inflammation

The pathologic changes in heart failure with preserved ejection fraction (HFpEF) occur in multiple steps: including cardiomyocyte hypertrophic remodeling and cell death, coronary microvascular rarefaction, and myocardial fibrosis caused by excessive functional activation of cardiac fibroblasts (CFs). Many recent studies focused on the importance of cardiac fibrosis in the pathogenesis of heart failure. Modulation of cardiac fibrosis has become an important target for failing hearts in postischemic cardiac remodeling and regeneration therapy. Improvement of left ventricular systolic/diastolic function in patients with HF was closely related to changes in myocardial collagen metabolism. Cardiac fibrosis is defined as a progressive accumulation of fibrillar extracellular matrix (ECM) in the myocardium. The regulation of ECM remodeling is primarily mediated by CFs, which are the main non-muscle cells in the heart. CFs modulate matrix turnover in non-pathological conditions, and their activity is greatly enhanced after an acute cardiac event or during chronic cardiovascular disease. The phenotypic transformation of fibroblasts into osteoblasts, stimulated by the renin-angiotensin system (RAS), leads to tissue calcification. It is known that angiotensin II (Ang II) is the main active peptide in the RAS, regulating cellular and physiological responses in the cardiovascular system. A lot of evidence has implicated Ang II in the progression of myocardial fibrosis. Ang II has been suggested to be a potent pro-fibrotic molecule. Increased serum levels of Ang II are seen in patients with cardiovascular diseases that are associated with myocardial fibrosis, including atherosclerosis, hypertension, cardiac hypertrophy, and heart failure. Rapid myocardial cellular infiltration is evident in animals infused with Ang II, which suggests that the cellular component has an effector function in the development of myocardial fibrosis.
It has been reported that FXa-dependent protease-activating receptor (PAR)-1 and PAR-2 cleavage might play a role in tissue fibrosis and remodeling. Previous experiments in our laboratory demonstrated that the activation of fibroblast cardiac fibrosis is stimulated by FXa signaling via activator protein (AP)-1 and the nuclear factor kappa-light-chain-enhancer of the activated B cell (NF-κB) pathway, accompanied by reactive oxygen species (ROS) elevation and fibrotic signal activation in growth-arrested fibroblasts. Factor Xa (FXa) inhibitors are anticoagulants to prevent and treat stroke and other thromboembolic events. Rivaroxaban, an approved oral direct FXa inhibitor, has been evaluated in a number of clinical settings to prevent and treat venous thromboembolism and stroke prophylaxis in atrial fibrillation (AF). However, there is a limited amount of evidence to substantiate the pleiotropic effects of direct FXa inhibitors. The aim of this study was to investigate whether rivaroxaban treatment attenuates cell proliferation and migration in Ang II induced mouse cardiac derived fibroblasts.

METHODS

Reagents: Rivaroxaban was obtained from Toronto Research Chemicals (Ontario, Canada). Ang II was purchased from Sigma-Aldrich (St. Louis, MO, USA). MTT assay kits were from Roche (Basel, Switzerland). Cell migration assay kits were obtained from Platypus Technologies, LLC (Madison, WI, USA). Mouse cytokine array kits and TIMP-1 ELISA kits were from R&D Systems (Minneapolis, MN, USA). Diff-Quick stain was obtained from SYSMEX (Hyogo, Japan). The Cignal Finder 45-pathway Reporter Array and Attractene were from Qiagen (Dusseldorf, Germany). The Dual-Luciferase Reporter Assay Systems were obtained from Promega Corp. (Madison, WI, USA). All other reagents used were of analytical grade.

Cell cultures: Cardiac derived fibroblasts were isolated from 1-day-old C57/BL/6 mice by the methods previously described with minor modification. In brief, the ventricles were minced and digested using a collagenase/pancreatin mixture at 37°C for 30 minutes. The digested fractions were filtered through a sterile 70 μm nylon mesh. The pooled cells were re-suspended in alpha-modified Eagle’s medium (α-MEM) with 10% fibroblast growth factor. The cells were incubated twice at 37°C for 30 minutes using different two flasks. The cells were maintained in α-MEM supplemented with 10% fetal calf serum (FCS). Unless stated otherwise, the cells were starved in Hank’s balanced salt solution (HBSS) and subsequently stimulated as described.

Rivaroxaban pretreatment: Rivaroxaban was dissolved in dimethyl sulfoxide (DMSO), and diluted 100 times with α-MEM with 1% FCS and 1% streptomycin. The final concentration of DMSO in culture media was 1%. CFs without rivaroxaban pretreatment (control) were treated with α-MEM with 1% FCS, 1% streptomycin, and 1% DMSO under the same conditions as rivaroxaban pretreatment.

Cell migration assay: Cell migration was determined using an Oris Pro Cell migration assay kit as described previously. CFs were pretreated with rivaroxaban (0.1 μg/mL) for 4 hours during adherence (n = 5). The cells were fixed and stained using Diff-Quick staining. Images were captured immediately and at 24 hours after Ang II stimulation (10^{-8} M). The valuation was taken as the area which a cell occupies using an imaging analysis system (Image J, National Institutes of Health, Bethesda, MD, USA).

Proliferation assay (MTT Assay): Cells seeded at a density of \(2.5 \times 10^4 \text{cells/cm}^2\) in 96-well plates were pretreated with or without rivaroxaban (5, 1, 0.1, and 0.01 μg/mL) for 4 hours, and then stimulated by Ang II (10^{-8} M) in 12 and 24 hours (n = 5). Cell proliferation was determined as the indicated intervals using MTT assay as described before.

Mouse cytokine array: Comprehensive cytokine and chemokine productions were evaluated by Mouse Cytokine Array Panel A Array after 18 hours of Ang II stimulation, as described previously. CFs were plated at 2.5 × 10^3 cells/mL in 96-well plates, pretreated either with or without rivaroxaban (0.1 μg/mL), and stimulated by Ang II (10^{-8} M). After the treatment, the supernatants were harvested and analyzed using the cytokine panel A array according to the manufacturer’s protocol. Briefly, the array membranes were blocked with blocking buffer at room temperature for 1 hour, and media and the detection antibody cocktail were added and incubated at 4°C overnight. After washing 3 times with 2 mL of wash buffer at room temperature, streptavidin-HRP was added to each membrane and incubated at room temperature for 30 minutes. After washing, the cytokines were detected by a chemiluminescence reaction. We exposed wrapped membranes to X-ray film for 4 minutes and analyzed the array image file using image analysis software.

TIMP-1 ELISA: After the pretreatment with rivaroxaban (0.01, 0.1, 1, and 5 μg/mL) for 4 hours, tissue inhibitors of metalloproteinases (TIMP)-1 production were evaluated by ELISA using the supernatant of mouse CFs after incubation with Ang II (10^{-7} M). The ELISA immunoassay was performed according to the manufacturer’s protocol. Briefly, the wells were precoated with a monoclonal antibody specific for TIMP-1. The standards, controls, and samples were added, and the samples were incubated for 2 hours. After washing, an enzyme-linked polyclonal antibody specific for TIMP-1 was added. After another wash, the samples were incubated with a substrate solution for 30 minutes, and the color reaction was stopped by the addition of a stop solution. The optical density of each well was measured immediately with a microplate reader at 450 nm.

Signal pathway reporter assay: The signal pathways were evaluated using the signal pathway reporter assay. CFs were seeded in 96-well cell culture plates and transfected with the signal reporter array panel of transcription factors. The transcription factors used for the 45 signaling pathways were the following: activating transcription factor (ATF)2/3/4, androgen receptor, nuclear factor (Nrf) 2 /Nrf1, ATF6, CCAAT-enhancer-binding proteins, cyclic adenosine monophosphate (cAMP) response enhancer-binding proteins, E2F, p53, early growth response protein 1, CBF/NF-Y/Y1, estrogen receptor, GATA, glucocorticoid receptor, heat shock transcription factor-1, metal regulatory transcription factor-1, Gli, hepatocyte nuclear factor-4, hypoxia inducible factor-1a, interferon regulatory factor-1, signal transducer and activator of transcription (STAT)1/2, STAT4, STAT1, Krippel-like factor 4, liver X receptor, serum response factor (SRF)/Elk-1, AP-1, monocyte enhancer factor-2, c-Myc, Nanog, recombining binding protein Suppressor of Hairless, NF-κB, octamer-binding transcription factor 4, Pax6, FOXO, nuclear factor of activated T cells, peroxisome
proliferator-activated receptor, progesterone receptor, retinoic acid receptor, retinoid X receptor, Sox2, SP1, STAT3, SMAD2/3/4, vitamin D receptor, TCF/LEF, and aryl hydrocarbon receptor. In each well, a pathway-specific transcription factor was transfected along with renilla luciferase (constitutively expressing the renilla construct, 20:1) using Attractene. Sixteen hours after the transfection, the cells were stimulated with Ang II (10\(^{-8}\) M), and then dual luciferase assays were performed as described previously.

Statistical analysis: All results represented experiments from a minimum of three separate preparations. Data are expressed as the mean ± SE unless otherwise indicated. Comparisons were performed using Student’s t test or a one-way analysis of variance followed by Bonferroni correction where appropriate. A P value of ≤0.05 was considered to be statistically significant.

RESULTS

Cell migration and proliferation: Cardiac fibroblast (CF) migration was evaluated 24 hours after the addition of Ang II. Only Ang II stimulation led to augmented cell migration in mouse CFs. Cell migration was significantly decreased by 73% in rivaroxaban induced cells compared to without rivaroxaban (Figure 1A). Rivaroxaban pretreatment by itself did not change the proliferation of cardiac fibroblasts (CFs). RX pretreatment itself did not change cell proliferation compared to cells without rivaroxaban at various times and concentrations. Rivaroxaban at concentrations of 0.01 μg/mL, 0.1 μg/mL, 1 μg/mL, and 5 μg/mL inhibited cell proliferation by 65%, 90%, 87%, and 80%, respectively, after 12 hours of Ang II stimulation. After 24 hours of stimulation, the corresponding values were 27%, 69%, 71%, and 69%, respectively. The inhibitory effect of rivaroxaban did not appear to be concentration-dependent (Figure 1B).

Mouse cytokine array: In a mouse cytokine array measuring 40 cytokines, comprehensive cytokine and chemokine productions after 18 hours of Ang II stimulation were decreased in rivaroxaban-pretreated CFs compared to cells without pretreatment. As shown in Figure 2, Ang II stimulation increased expression of 8 inflammatory mediators; chemokine (C-C motif) ligand 1 (I-309), interleukin(IL)-16, chemokine (C-X-C motif) ligand 11 (I-TAC), macrophage colony-stimulating factor (M-CSF), monocyte chemotactic protein-5 (MCP-5), stromal cell-derived factor-1 (SDF-1), TIMP-1, and TNF-α. The productions of these 8 cytokines were significantly reduced with rivaroxaban pretreatment. Increasing rates of cytokine chemiluminescence reactions due to Ang II stimulation and the inhibition rates of rivaroxaban are presented in the Table. TIMP-1 chemiluminescence reactions in particular were extremely high and revealed a high inhibitory effect of rivaroxaban.

Figure 1. A: Cell migration after 24 hours of stimulation by Ang II, with or without RX pretreatment (0.1 μg/mL). Confluent CFs were pretreated with or without RX for 4 hours, the Oris’s stoppers were removed, and growth-arrested CFs were stimulated with Ang II. Images were captured immediately after adherence at 0 hours (a) and 24 hours (b-d) after Ang II stimulation. Scale bars = 500 μm. (a) just after adherence, 0 hours after stimulation. b: control in 24 hours. c: only Ang II stimulation in 24 hours. d: Ang II stimulation after RX pretreatment in 24 hours. The bar graph represents the average data, expressed as the cell gross area per 5 fields (e). The data are expressed as the mean ± SE of 3 independent experiments. *P < 0.05. B: Rivaroxaban (RX) pretreatment reduced the cell proliferation of cardiac fibroblasts (CFs). RX pretreatment itself did not change the cell proliferation compared with the control group [without RX and angiotensin (Ang) II treatment]. CFs without rivaroxaban pretreatment. After RX pretreatment (0.01-5 μg/mL), cell proliferation was stimulated for 12 and 24 hours with Ang II (10\(^{-8}\) M). The results are shown as the mean ± SE of 3 independent experiments. *P < 0.05.

Figure 2. Representative figure of mouse cytokine array. Three independent experiments were duplicated. Rivaroxaban (RX) attenuated comprehensive cytokine and chemokine production after 18 hours of angiotensin (Ang) II (10\(^{-8}\) M) stimulation in cardiac fibroblasts (CFs). From 40 cytokines, chemokine (C-C motif) ligand 1 (I-309), chemokine (C-X-C motif) ligand 11 (I-TAC), macrophage colony-stimulating factor (M-CSF), monocyte chemotactic protein-5 (MCP-5), stromal cell-derived factor-1 (SDF-1), tissue inhibitor of metalloproteinase-1 (TIMP-1), and tumor necrosis factor-α (TNF-α) were significantly reduced with RX pretreatment. The results of 3 independent experiments are shown in the Table.
RIVAROXABAN INHIBITS FIBROTIC PROGRESSION IN VITRO

TIMP-1 production: TIMP-1 production is the key event in the pathogenesis of fibrosis and is known to be deeply associated with cardiac fibrosis to cause heart failure. Ang II (10^{-8} M) stimulation significantly increased TIMP-1 levels in the supernatant of cultured CFs (66% increase, \( P < 0.05 \)). As shown in Figure 3, TIMP-1 levels were decreased by rivaroxaban pretreatment in Ang II-induced CFs compared to cells without rivaroxaban at various concentrations (decreases of 35%, 47%, 47%, and 57% at rivaroxaban concentrations of 0.01, 0.1, 1, and 5 \( \mu g/mL \)).

Signal transduction of fibroblast activation: We identified a set of transcription factors potentially involved in the effects of rivaroxaban on Ang II-induced cardiac fibroblasts using the luciferase reporter system (Figure 4). In rivaroxaban pretreated cells, cAMP response element binding protein (CREB), SRF/Elk-1, NF-\( \kappa \)B, and AP-1 were significantly decreased by 78%, 75%, 82%, and 78%, respectively (\( P < 0.05 \)) compared to those without rivaroxaban. CREB is an essential pathway of Ang II and TNF-\( \alpha \), and SRF/Elk-1, which represents mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway. Both CREB and SRF/Elk-1 are downstream transcription factors of MAPK/ERK. The NF-\( \kappa \)B and AP-1 pathways and their cross talk with MAPK/ERK may play roles in the fibroproliferative activation in Ang II-induced CFs.

Discussion

This study has demonstrated that rivaroxaban reduces cell migration and proliferation by reducing inflammatory signal pathways including NF-\( \kappa \)B, in Ang II-induced mouse cardiac fibroblasts. In the present study, the concentrations of rivaroxaban that had a significant effect on the inhibition of Ang II-induced CFs activation were 0.1 to 5 \( \mu g/mL \).

Rivaroxaban, a direct factor Xa inhibitor, is an oral anticoagulant approved for stroke prevention in patients with non-valvular AF and peripheral artery disease, and has also

Table. Chemiluminescence Reactions of Inflammatory Mediators by Mouse Cytokine Array

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Ang II</th>
<th>RX + Ang II</th>
<th>Control</th>
<th>Increasing rate (times)</th>
<th>Inhibition rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-309, dpi</td>
<td>346 ± 11</td>
<td>135 ± 10</td>
<td>112 ± 23</td>
<td>3.1</td>
<td>90</td>
</tr>
<tr>
<td>Interleukin-16, dpi</td>
<td>342 ± 13</td>
<td>131 ± 21</td>
<td>105 ± 11</td>
<td>3.3</td>
<td>89</td>
</tr>
<tr>
<td>I-TAC, dpi</td>
<td>331 ± 26</td>
<td>38 ± 15</td>
<td>29 ± 11</td>
<td>11.4</td>
<td>97</td>
</tr>
<tr>
<td>M-CSF, dpi</td>
<td>638 ± 38</td>
<td>228 ± 99</td>
<td>192 ± 23</td>
<td>3.3</td>
<td>92</td>
</tr>
<tr>
<td>MCP-5, dpi</td>
<td>373 ± 18</td>
<td>95 ± 23</td>
<td>91 ± 20</td>
<td>4.1</td>
<td>98</td>
</tr>
<tr>
<td>SDF-1, dpi</td>
<td>1070 ± 172</td>
<td>520 ± 55</td>
<td>255 ± 30</td>
<td>4.2</td>
<td>67</td>
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<tr>
<td>TIMP-1, dpi</td>
<td>5128 ± 550</td>
<td>2549 ± 297</td>
<td>2431 ± 132</td>
<td>2.1</td>
<td>96</td>
</tr>
<tr>
<td>TNF-( \alpha ), dpi</td>
<td>309 ± 23</td>
<td>66 ± 28</td>
<td>55 ± 5</td>
<td>5.6</td>
<td>96</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SE. Ang II indicates angiotensin II; RX, rivaroxaban; I-309, chemokine (C-C motif) ligand 1; I-TAC, chemokine (C-X-C motif) ligand 11; M-CSF, macrophage colony-stimulating factor; MCP-5, monocyte chemotactic protein-5; SDF-1, stromal cell-derived factor-1; TIMP-1, tissue inhibitor of metalloproteinase-1; and TNF-\( \alpha \), tumor necrosis factor-\( \alpha \). Increasing rates were evaluated by comparing control groups to Ang II stimulation groups. *Inhibition rates for rivaroxaban, compared with Ang II stimulation groups.
been approved in Europe to prevent recurrent ischemic events in patients with recent acute coronary syndromes.\textsuperscript{25} It also has been reported that rivaroxaban attenuates progression and promotes stability of advanced atherosclerotic lesions in hyperlipidemic apolipoprotein E-deficient mice.\textsuperscript{26}

Ang II is the main active peptide in the RAS, regulating cellular and physiological responses in the cardiovascular system.\textsuperscript{8} Tissue culture and animal experiments have demonstrated a variety of mechanisms by which Ang II, an effector of the RAS, may promote atherogenesis by induction of cell growth and proliferation, oxidative stress, adhesion, and chemoattractant molecules and cytokines. Ang II stimulates the production of ROS by inducing nicotinamide adenine dinucleotide oxido-dase.\textsuperscript{27} The enzymatically active flavoprotein subunit gp-91phox of nicotinamide adenine dinucleotide phosphate oxidase present on fibroblasts is chiefly responsible for Ang II-stimulated oxidative stress \textit{in vivo}.\textsuperscript{28} In addition, ROS activated coagulant tissue factor (TF).\textsuperscript{29} Active TF binds factors VIIa and Xa, and cleaves and activates PAR-2, and soluble Xa mediates PAR1.\textsuperscript{30,31} By blocking PAR-1 and PAR-2 with rivaroxaban, the production of several inflammatory cytokines, such as TIMP-1, IL-16, IL-6, 1-TAC, M-CSF, MCP-5, SDF-1, and TNF-\alpha, were significantly inhibited in Ang II-induced CFs. These inflammatory mediators are expected to accelerate cell migration and proliferation in activated CFs. Moreover, these mediators are also known to activate transformations of CFs to various types of cells in the heart. Among these 9 cytokines, TIMP-1, IL-16, and TNF-\alpha are reported to be deeply associated with cardiac fibrosis. It has been also reported that TIMP-1 and TNF-\alpha through growth stimulating signal pathways, such as the Wnt and NF-xB pathways, are associated with tissue calcification.\textsuperscript{6}

TIMP-1 is a natural glycoprotein that can inhibit the activation of matrix metalloproteinases (MMPs).\textsuperscript{32} A manifestation of myocardial remodeling is an increase in ECM which results from dysregulated levels of collagen synthesis, and its degradation via the delicate balance maintained between matrix MMPs and TIMPs.\textsuperscript{33} Increased protein levels of TIMP-1 were observed in both hypertrophied and unloaded hearts, suggesting the influence of changes in TIMPs in cardiac remodeling.\textsuperscript{35} Adenoviral overexpression of TIMP-1 in cultured cardiac fibroblasts revealed that TIMP-1 induced fibroblast proliferation and provoked a switch to a more activated myofibroblast phenotype.\textsuperscript{34} TIMPs may stimulate cardiac fibroblast proliferation and phenotypic differentiation into myofibroblasts at the site of tissue injury, thereby contributing to the formation of a qualitative and mature collagen matrix.\textsuperscript{37} Reduction of TIMP-1 production by rivaroxaban pretreatment may cause not only proliferative inhibition but also suppress phenotypic change in Ang II-induced CFs.

We have previously reported that the activation of cardiac fibroblasts is stimulated by FXa signaling via AP-1 and NF-xB pathways, accompanied by increasing ROS production and fibrotic signal activation through the ERK and JNK pathways.\textsuperscript{14} Substances such as ROS are capable of activating intracellular transcription factors such as AP-1 and NF-xB. The MAPK/ERK pathways also activate AP-1 and NF-xB, eventually inducing TIMP-1 expression.\textsuperscript{38} Additionally, TIMP-1 is upregulated by transcription factors including MAPK modulation.\textsuperscript{39} In the present study, we also demonstrated that rivaroxaban inhibits all of these inflammatory signal pathways, including AP-1, NF-xB, and MAPK/ERK (Figure 5).

IL-16 has been shown to be a key mediator of several inflammatory, allergic, or infectious diseases.\textsuperscript{36-40} IL-16 was one of the first cytokines characterized with chemoattractant activity for human T cells and therefore was originally designated as lymphocyte chemoattractant factor. IL-16 can stimulate the synthesis of proinflammatory cytokines, including IL-1, IL-6, and TNF-\alpha in monocytes.\textsuperscript{41} IL-16 has been reported to mediate cardiac inflammation leading to progressive cardiac fibrosis and LV stiffness.\textsuperscript{42} However, the signal pathways inducing IL-16 still remain unclear. On the other hand, TNF-\alpha is known to be involved in cardiac fibrosis and remodeling. TNF-\alpha evokes inflammation and may synergically activate IL-16-related signaling cascade in CFs mainly through the NF-xB.\textsuperscript{43}

In clinical settings, in addition to its classical role in the prevention or treatment of thromboembolic diseases, rivaroxaban was also evaluated in a phase III clinical trial in patients stabilized after acute coronary syndromes.\textsuperscript{39} From the analysis of the ROKET AF trial, a 14% reduction in the hazard for cardiovascular death or acute coronary syndrome was observed in patients without prior myocardial infarction taking rivaroxaban compared with that of warfarin.\textsuperscript{44} This report suggests that rivaroxaban has an effect on the early phase of development of atherosclerosis. Although no report has demonstrated the treatment effect of rivaroxaban in heart failure (HF) directly, it was reported the clinical outcomes of rivaroxaban were similar in patients with and without HF, suggesting the efficacy and safety of rivaroxaban extend to the patient population with HF and AF.\textsuperscript{45} Our data in the present \textit{in vitro} study may support these clinical effects of rivaroxaban in cardiovascular disease. In the next stage of the study, a pressure overload animal experimental model of HF could be effective to confirm the cardioprotective and anti-fibroproliferative effect of rivaroxaban on HFpEF. Rivaroxaban reduced angiotensin II-induced cardiac fibroblast migration and proliferation, accompanied by reductions in the
production of various inflammatory cytokines. In the present study, we have demonstrated the novel pleiotropic effects of the direct Xa inhibitor rivaroxaban in mouse cardiac derived fibroblasts.

**DISCUSSION**

**Conflict of interest:** Dr. Minako Yamaoka-Tojo was partly supported by international grants from MSD K.K., Bayer Pharma, Daichi-Sankyo, and Boehringer Ingelheim. Dr. Junya Ako received speaking honorarium from Bayer Pharma. Other authors have nothing to disclose regarding this manuscript.

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