Cardioprotective Effects of Rivaroxaban on Cardiac Remodeling After Experimental Myocardial Infarction in Mice

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Background: Direct-activated factor X (FXa) plays an important role in thrombosis and is also involved in inflammation via the protease-activated receptor (PAR)-1 and PAR-2 pathway. We hypothesized that rivaroxaban protects against cardiac remodeling after myocardial infarction (MI).

Methods and Results: MI was induced in wild-type mice by permanent ligation of the left anterior descending coronary artery. At day 1 after MI, mice were randomly assigned to the rivaroxaban and vehicle groups. Mice in the rivaroxaban group were provided with a regular chow diet plus rivaroxaban. We evaluated cardiac function by echocardiography, pathology, expression of mRNA and protein at day 7 after MI. Rivaroxaban significantly improved cardiac systolic function, decreased infarct size and cardiac mass compared with the vehicle. Rivaroxaban also downregulated the mRNA expression levels of tumor necrosis factor-α, transforming growth factor-β, PAR-1 and PAR-2 in the infarcted area, and both A-type and B-type natriuretic peptides in the non-infarcted area compared with the vehicle. Furthermore, rivaroxaban attenuated cardiomyocyte hypertrophy and the phosphorylation of extracellular signal-regulated kinase in the non-infarcted area compared with the vehicle.

Conclusions: Rivaroxaban protected against cardiac dysfunction in MI model mice. Reduction of PAR-1, PAR-2 and proinflammatory cytokines in the infarcted area may be involved in its cardioprotective effects.

Key Words: Cardiac remodeling; Myocardial infarction; Protease-activated receptor (PAR)-1; PAR-2; Rivaroxaban

Cardiac remodeling is an important prognostic factor in heart failure (HF). Myocardial infarction (MI) is the most common cause of HF and our understanding of the effect of cardiac remodeling in HF is based on MI studies. The pathology of MI suggests that the inflammatory changes in the infarcted tissue correlate closely with cardiac function and prognosis after MI. These findings suggest that modulation of the inflammatory response in the infarcted myocardium could potentially improve cardiac remodeling after MI.

Rivaroxaban, an oral anticoagulant, is used to prevent and treat atrial fibrillation and venous thrombosis, because it exerts its anticoagulant properties by inhibiting activated factor X (FXa). It has been reported that combination therapies that include low-dose rivaroxaban seem to be effective in secondary prevention of acute coronary syndrome, which suggests that rivaroxaban is effective not only for atrial fibrillation and venous thrombosis but also for coronary artery thrombosis. However, there is insufficient evidence about the cardioprotective effects of rivaroxaban on cardiac remodeling after MI.

Rivaroxaban also exhibits anti-inflammatory properties, acting through amelioration of protease-activated receptor (PAR)-1 and -2 in arteriosclerosis models. In addition, basic in vivo and in vitro research work has examined in detail the relationship between PARs and cardiac remodeling.
Effects of Rivaroxaban on MI

Experimental Protocol
MI was induced by permanent ligation of the left anterior descending coronary artery at the level of the left atrium in anesthetized mice, as described in detail previously.

Methods
Animals
Male C57BL/6J (WT) mice were purchased from CLEA Japan Inc. (Tokyo, Japan). All mice were housed under a 12–12-h light–dark cycle and used for experiments between 9 and 12 weeks of age. Animal procedures were approved by the Animal Care and Use Committee of Kumamoto University, and conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (publication No. 85-23, revised 1996).

Using wild-type (WT) and PAR-2-deficient mice, a recent experimental study showed that rivaroxaban suppressed cardiac remodeling after MI, although the detailed underlying molecular mechanism of rivaroxaban was not demonstrated in the study.

Based on these observations, we hypothesized that rivaroxaban might contribute to cardiac remodeling after MI through the PAR-1 and PAR-2 pathways.

Results

Figure 1. Experimental protocol. CAL, coronary artery ligation; %FS, percent fractional shortening; UCG, ultrasound cardiography.

Figure 2. Effect of rivaroxaban on left ventricular (LV) remodeling after myocardial infarction (MI). Changes in (A) LV end-diastolic diameter (LVDd), (B) LV systolic dimension (LVDs), (C) M-mode percent fractional shortening (%FS), (D) interventricular septum thickness in diastole (IVSTD), and (E) posterior LV wall thickness in diastole (PLWTD) assessed by echocardiography before and at days 1, 7 and 14 after MI in the rivaroxaban and vehicle groups (n=35/group). Data are mean±standard error. *P<0.05 vs. vehicle.
containing EDTA-2Na (Terumo, Tokyo) and automatically analyzed using an ADVIA®2120i hematology system (Siemens Healthcare, Germany).

Histology and Immunohistochemistry
The harvested hearts were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. To determine the size of the MI-related scar, each sample at day 7 after MI was divided into 5 transverse sections from the apex to the base of the LV. The sections were stained with Masson trichrome to determine the size of the MI. The total circumference of the infarcted area was divided by the total LV circumference. Infarct length and total LV circumference were measured along the endocardial and epicardial surfaces in each section. We analyzed cardiomyocyte cross-sectional area as described previously. Immunohistochemistry was performed to determine the presence and type of inflammatory cells. Neutrophils and macrophages in the infarcted and non-infarcted areas were identified by staining with anti-Gr-1 (Southern Biotechnology, 1900-01) and anti-Iba1 (Wako Pure Chemical Industries, 019-19741), respectively. The number of positive cells was counted in 3 different fields. All measurements were performed with Image J software (National Institute of Health, Bethesda, MD, USA).

RT-PCR Assay
We evaluated the mRNA expression by RT-PCR, using the procedure described in detail previously. Briefly, total RNA was extracted from the heart tissue at day 7 after MI using the RNA Easy Mini Kit (Qiagen, Hilden, Germany). The cDNA was synthesized using the T-PCR System 2700 (Applied Biosystems). RT-PCR was performed using a TaqMan Universal Master Mix kit with a CFX384 Real-Time System (Bio-Rad, Hercules, CA). We measured the

Blood Sampling
It was assumed that rivaroxaban could potentially increase the likelihood of bleeding events. For this reason, we compared hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), and mean corpuscular Hb (MCH) between the rivaroxaban and placebo groups at day 14 after MI. Blood was collected from the right atrium into a tube containing EDTA-2Na (Terumo, Tokyo) and automatically analyzed using an ADVIA®2120i hematology system (Siemens Healthcare, Germany).

HealthCare, Germany) or placebo group. All mice were provided with regular chow diet before randomization. After randomization, mice in the rivaroxaban group were provided with regular chow diet including rivaroxaban (2.4g rivaroxaban/kg chow). The dietary dose of rivaroxaban in mice after MI was 138.5±50.3 mg/kg/day per mouse.

To determine the effect of treatment on cardiac function, echocardiography was repeated at day 7 and 14 after MI. Mice were anesthetized and the hearts were harvested for evaluation of mRNA and protein expression levels in the infarcted and non-infarcted regions by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay and western blot analysis at day 7 after MI. Furthermore, we measured the infarct size and assessed the extent of inflammatory cell infiltration by histopathological analysis at day 7 after MI. The study protocol is shown in Figure 1.

Echocardiography Analysis
Each mouse was anesthetized with isoflurane and echocardiography was performed while the animal breathed spontaneously. We evaluated the M-mode percent fractional shortening (%FS), left ventricular (LV) diastolic dimension (LVDd), LV systolic dimension (LVDs), interventricular septum thickness in diastole (IVSTd), and posterior LV wall thickness in diastole (PLWtd) using the Xario system (Toshiba, Tokyo, Japan) with a 12-MHz linear array transducer.

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mRNA levels of A-type natriuretic peptide (ANP, GenBank Acc: NM_008725.3), B-type natriuretic peptide (BNP; NM_008726.5), collagen type 1 alpha1 (Col1α1; NM_007742.4), collagen type 3 alpha1 (Col3α1; NM_009930.2), interleukin-1 beta (IL-1β; NM_008361.4), interleukin-6 (IL-6; NM_031168.2), monocyte chemoattractant protein-1 (MCP-1; NM_011333.3), matrix metalloproteinase-2 (MMP-2; NM_008610.2), MMP-9 (NM_013599.4), transforming growth factor-beta1 (TGF-β1; NM_011577.2), tissue inhibitor of metalloproteinase-1 (TIMP-1; NM_001044384.1), tumor necrosis factor-alpha (TNF-α; NM_013693.3), PAR-1 (NM_010169.3), PAR-2 (NM_007974.4), PAR-3 (NM_010170.4), and PAR-4 (NM_007975.4). The mRNA expression level was expressed relative to the expression level of the control (endogenous 18S ribosomal RNA gene).

**Western Blot Analysis**

Western blot analysis was performed with the SDS-PAGE Electrophoresis System, as described previously. We used primary antibodies against phosphorylated extracellular signal-regulated kinase (p-ERK), ERK and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Danvers, MA, USA), in combination with a peroxidase-conjugated secondary antibody. The protein bands were detected with chemiluminescence. An ImageQuant LAS 4000 minibiomolecular imager (Fujifilm, Tokyo) was used for quantification of the band density.

**Figure 4.** Characterization of inflammatory response at day 7 after myocardial infarction (MI). (A) Representative images of immunohistochemical staining with Gr-1 and Iba-1 of LV cross-sections in the vehicle- and rivaroxaban-treated mice at day 7 after MI. Scale bars=50μm (B) Density of Gr-1-positive macrophages and Iba-1-positive granulocytes in the infarcted area of the LV in vehicle- and rivaroxaban-treated mice at day 7 after MI. Data is mean±standard error (n=7/group). LV, left ventricle.
Results

Effects of Rivaroxaban on Echocardiographic Parameters and Bleeding Complications After MI

Figure 2 shows the echocardiographic parameters measured before MI and at day 1, 7 and 14 after MI. There were no significant differences between the 2 treatment groups in LVDd, LVDs, %FS, IVSTd, or PLWTd on the day before MI and the day after MI. However, LVDd, %FS, and IVSTd were significantly improved at day 7 and 14 after MI in the rivaroxaban group compared with the vehicle group, but there were no significant differences in LVDd and PLWTd between groups. In the analysis for serial changes in the echocardiographic parameters, 2 of 35 and 4 of 35 mice died in the vehicle and rivaroxaban groups.
**Figure 6.** Effect of rivaroxaban on PAR-1 and PAR-2 mRNA expression in the infarcted area at day 7 after myocardial infarction (MI). Quantitative results of real-time reverse transcriptase PCR for (A) PAR-1, (B) PAR-2, (C) PAR-3, and (D) PAR-4 mRNA levels at day 7 after MI. The mRNA level was expressed relative to the level of endogenous control 18S ribosomal RNA (n=15/group). See Figure 4 for explanation of the box-and-whisker plots. PAR, protease-activated receptor.

**Figure 7.** Effect of rivaroxaban on cardiomyocyte hypertrophy and phosphorylation of extracellular signal-regulated kinase (ERK) in the non-infarcted area. (A) Representative images of the cardiomyocyte cross-sectional area. Scale bars=50 μm. (B) Quantitative analysis of cardiomyocyte cross-sectional area in vehicle and rivaroxaban mice at day 7 after MI (n=7/group). Data are mean±standard error. (C) Representative images of western blot analysis of the phosphorylation of ERK at day 7 after MI. (D) Quantitative analysis of the immunoblots of phosphorylation of ERK at day 7 after MI (n=6/group).
Histomorphometric and Immunohistochemical Analysis at Day 7 After MI
Because LVDs, %FS, and IVSTd improved significantly at day 7 after MI, we conducted histomorphometric analysis using samples obtained at day 7 after MI. As shown in Figure 3A, Masson’s trichrome staining demonstrated myocardial fibrosis in the infarcted areas in both groups of mice; however, the infarct size was significantly smaller in the rivaroxaban group compared with the vehicle group (Figure 3B). Furthermore, rivaroxaban significantly attenuated heart weight/tibial length compared with the vehicle group at day 7 after MI (Figure 3C).

Discussion
In this study, we showed that the cardiac remodeling process after MI improved with administration of rivaroxaban. Although it has been reported that rivaroxaban improves cardiac function through inhibition of PAR-2,22 the exact underlying mechanism of such cardioprotective effects remains unknown. The present study extended previous observations of the cardioprotective effects of rivaroxaban after experimental MI by demonstrating that rivaroxaban protected against cardiac dysfunction in MI model mice probably by reducing the mRNA levels of PAR-1, PAR-2 and proinflammatory cytokines in the infarcted area.

Pars are a family of protease-mediated G protein-coupled 7 transmembrane receptors and so far 4 (PAR-1, PAR-2, PAR-3 and PAR-4) have been identified.30,31 PAR-2 is activated by factor Xa, whereas PAR-1 is activated by not only factor Xa but also thrombin.30 In this study, we found no significant differences in PAR-3 or PAR-4, compared with the vehicle, in the infarcted area at day 7 after MI; however, rivaroxaban significantly downregulated the mRNA expression of PAR-1, PAR-2, TNF-α and TGF-β. Using echocardiography, the present study demonstrated that rivaroxaban prevented excessive thinning of the IVSTd and improved cardiac function compared with the vehicle. In addition, pathological analysis demonstrated that rivaroxaban significantly reduced infarct size and heart weight. Previous studies using experimental MI models have demonstrated that inhibition of PAR-1 or PAR-2 downregulated the expression of proinflammatory cytokines and various markers of myocardial fibrosis.18,22 Furthermore, in several experimental models of atherosclerosis, atrial fibrosis and ischemic cardiomyopathy, rivaroxaban also suppressed proinflammatory cytokines.16,18,34 Based on these findings, it is conceivable that the cardioprotective effects of rivaroxaban are mediated through inhibition of PAR-1 and PAR2 in the infarcted area.

Various inflammatory reactions are associated with cardiac remodeling after MI.35-42 Hara et al reported that stimulation of mice macrophages with FXa resulted in increases in inflammatory cytokines, and such increase was suppressed by rivaroxaban administration.40 Other groups have demonstrated that rivaroxaban inhibits angiotensin II-induced functional activation in cultured cardiac fibroblasts,43,44 and that rivaroxaban reduced the number of apoptotic cardiomyocytes and decreased the mRNA expression of proinflammatory cytokines.34 In the present study, rivaroxaban interestingly reduced both TNF-α and TGF-β mRNA levels in the infarcted area.

Although TGF-β is known as a fibrotic factor,45 it is also known as a modulator of proinflammatory cytokines, such as TNF-α.46 In order to elucidate the role of TGF-β post-MI, further study will be needed to examine whether direct inhibition of TGF-β expression reflects the antifibrotic effect in the infarcted area after MI in rivaroxaban-treated and vehicle mice.

In the present study, there was no significant difference in the infiltration of inflammatory cells in the infarcted area between the rivaroxaban and vehicle groups, but rivaroxaban reduced the expression of proinflammatory cytokines in the infarcted area. Regarding this point, previous reports

Cardioprotective Effects of Rivaroxaban on Myocardial Gene Expression of Inflammatory Markers After MI
To investigate the mechanisms of rivaroxaban-induced improvement of cardiac function after MI, we evaluated the mRNA expression levels of cytokines involved in cardiac inflammation, tissue degradation, and fibrosis in the infarcted area at day 7 after MI. The mRNA expression levels of TNF-α and TGF-β in the infarcted area and those of ANP and BNP in the non-infarcted area were significantly lower in the rivaroxaban group compared with the vehicle group (TNF-α, P=0.015; TGF-β, P=0.033; ANP, P=0.008; BNP, P=0.045). On the other hand, the mRNA levels of MMP-2, MMP-9, TIMP-1, Colla1, Col3a1, IL-1β, IL-6, and MCP-1 in the infarcted and non-infarcted area were almost identical between treatment groups (Figure 5).

As shown in Figure 6, PAR-1 and PAR-2 mRNA expression levels in the infarcted area, but not in the non-infarcted areas, were significantly lower in the rivaroxaban group compared with the vehicle group at day 7 after MI (PAR-1, P=0.008; PAR-2, P=0.037). Neither the pathological process of MI nor rivaroxaban had any effect on the mRNA expression levels of PAR-3 and PAR-4.

Effect of Rivaroxaban in the Non-Infarcted Myocardium
Because rivaroxaban improved cardiac dysfunction and reduced heart weight/tibial length compared with the vehicle group, we analyzed cardiomycocyte cross-sectional area in the myocardial sections at day 7 after MI. Figure 7A and Figure 7B show that rivaroxaban statistically attenuated cardiomycocyte hypertrophy in the non-infarcted area compared with the vehicle group at day 7 after MI.

To determine the molecular mechanisms of the cardioprotective effects of rivaroxaban, we performed western blot analysis for phosphorylation of ERK. The results demonstrated significant decreases in p-ERK in the non-infarcted area (P=0.015), but not in the infarcted area at day 7 after MI in the rivaroxaban group compared with the vehicle group (Figure 7C-D).

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have shown that stimulated macrophages, fibroblasts and cardiomyocytes express PAR-1 and PAR-2, and that rivaroxaban inhibited the activation of these cells through the suppression of PAR-1 and PAR-2. 16,34,43,44 It is possible that rivaroxaban could downregulate the activation of inflammatory cells and ischemic cardiomyocytes in the infarcted area by reducing the levels of PAR-1 and PAR-2, and then improve cardiac remodeling after MI, even though the numbers of inflammatory cells in the infarcted area were identical between the 2 treatment groups in the present study.

It is well known that MI leads to fibrosis and thinning in the infarcted area, which also induces compensated cardiac remodeling in the non-infarcted area and leads to cardiomyocyte hypertrophy of the non-infarcted area by overload pressure. 48 In the present study, rivaroxaban improved cardiac function and infarct size compared with the vehicle group. Analysis of cardiomyocyte cross-sectional area in the myocardial sections of the non-infarcted area showed that cardiomyocyte hypertrophy in response to MI was smaller in the rivaroxaban group than in the vehicle group. Furthermore, rivaroxaban reduced the mRNA expression levels of ANP and BNP mRNA expression levels and p-ERK in the non-infarcted area. Previous studies identified that increased ANP and BNP levels reflected cardiac function and infarct size compared with the vehicle group. Analysis of cardiomyocyte cross-sectional area in the myocardial sections of the non-infarcted area showed that cardiomyocyte hypertrophy in response to MI was smaller in the rivaroxaban group than in the vehicle group. Furthermore, rivaroxaban reduced the mRNA expression levels of ANP and BNP mRNA expression levels and p-ERK in the non-infarcted area. Previous studies identified that increased ANP and BNP levels reflected cardiac function and infarct size compared with the vehicle group. Analysis of cardiomyocyte cross-sectional area in the myocardial sections of the non-infarcted area showed that cardiomyocyte hypertrophy in response to MI was smaller in the rivaroxaban group than in the vehicle group. Furthermore, rivaroxaban reduced the mRNA expression levels of ANP and BNP mRNA expression levels and p-ERK in the non-infarcted area. Previous studies identified that increased ANP and BNP levels reflected cardiac function and infarct size compared with the vehicle group. Analysis of cardiomyocyte cross-sectional area in the myocardial sections of the non-infarcted area showed that cardiomyocyte hypertrophy in response to MI was smaller in the rivaroxaban group than in the vehicle group. Furthermore, rivaroxaban reduced the mRNA expression levels of ANP and BNP mRNA expression levels and p-ERK in the non-infarcted area. Previous studies identified that increased ANP and BNP levels reflected cardiac function and infarct size compared with the vehicle group.

Study Limitations
First, the follow-up was limited to day 14 after MI, so the long-term effects of rivaroxaban on the myocardium after MI remain to be defined. Second, we did not analyze a PAR-1 and PAR-2 gain-of-function MI model. Previous reports demonstrated that PAR-1 overexpression induces eccentric hypertrophy and dilation. 19 In addition, PAR-2 overexpression also induces cardiac hypertrophy, fibrosis and inflammation. 19 Furthermore, myocardial ischemia–reperfusion leads to increased PAR-1 and PAR-2 expression, and both PAR-1 knockout mice and PAR-2 knockout mice had significant cardioprotective effects compared with WT mice in the same model. 19,21 Third, we did not perform in vitro studies to confirm the cardioprotective effects of rivaroxaban in the MI model. Regarding this point, previous studies using cultured and stimulated macrophages, fibroblasts and cardiomyocytes have already described the anti-inflammatory and antifibrotic effects of rivaroxaban. 16,34,43,44 We speculated that rivaroxaban might suppress the activation of these cells and regulate the inflammatory and fibrotic response in the infarcted lesions post-MI.

In conclusion, the present study showed that rivaroxaban protected against cardiac dysfunction in MI model mice. Rivaroxaban could be potentially beneficial for improvement of cardiac remodeling after MI.

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IRB Information

The animal care and user committee of Kumamoto University granted an exemption from requiring ethics approval.

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Supplementary Files
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