Long-Term Treatment With a Specific Rho-Kinase Inhibitor Suppresses Cardiac Allograft Vasculopathy in Mice

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Abstract—Cardiac allograft vasculopathy (CAV) continues to be a major cause of late graft failure after cardiac transplantation. We have demonstrated that Rho-kinase, an effector of the small GTPase Rho, plays an important role in the pathogenesis of arteriosclerosis. In this study, we examined whether the Rho-kinase-mediated pathway is also involved in the pathogenesis of CAV using a specific Rho-kinase inhibitor and a dominant-negative Rho-kinase. Hearts from AKR mice were heterotopically transplanted to C3H/He (allograft) or AKR mice (isograft), and the effects of long-term oral treatment with fasudil, which is metabolized to a specific Rho-kinase inhibitor hydroxyfasudil, on CAV were examined at 2 and 4 weeks after the transplantation. Coronary remodeling in the allografts characterized by intimal thickening and perivascular fibrosis was dose-dependently suppressed in the fasudil group compared with the control group (P<0.01, n=9 to 10). The inhibitory effects of hydroxyfasudil were mimicked by in vivo gene transfer of dominant-negative Rho-kinase (P<0.05, n=4). Among the proinflammatory cytokines examined, those of macrophage migration inhibitory factor, interferon-γ, and transforming growth factor-β1 were upregulated in the control group and were dose-dependently inhibited in the fasudil group (P<0.01, n=5). Vascular inflammation in the allografts, as evidenced by accumulation of inflammatory cells (macrophages and T cells), was also significantly inhibited in the fasudil group (P<0.05, n=5 to 10). These results indicate that long-term treatment with fasudil suppresses CAV in mice, suggesting that Rho-kinase is an important therapeutic target for the prevention of CAV. (Circ Res. 2004;94:46-52.)

Key Words: arteriosclerosis ■ cytokines ■ transplantation

Cardiac allograft vasculopathy (CAV) continues to be a serious problem for long-term survival of patients with cardiac transplantation, as it is a major cause of the graft failure after the first year of transplantation.1-3 The coronary remodeling associated with CAV is characterized by progressive intimal thickening.4,5 Although the cause of CAV is known to be autoimmune, its pathogenesis, including the nature and sequence of cellular/molecular events leading to it, remains to be elucidated. To develop an effective preventive therapy for CAV, it is important to identify the key molecule(s) involved in this disorder.

Rho-kinase, an effector of small GTPase Rho, plays an important role in adhesion, migration, proliferation, and cytokinesis of vascular smooth muscle cells (VSMCs),6-8 all of which may be involved in the pathogenesis of arteriosclerosis. We have recently demonstrated that Rho-kinase is substantially involved in the pathogenesis of cardiovascular remodeling.6,9 Indeed, Rho-kinase is involved in migration of inflammatory cells, which may be involved in the pathogenesis of CAV.6,9 Rho-kinase also is substantially involved in the downregulation of endothelial NO synthase (eNOS).10

The present study was thus designed to examine whether Rho-kinase is involved in the pathogenesis of CAV in mice and, if so, what mechanisms are involved.

Materials and Methods

This study was reviewed by the Committee on Ethics in Animal Experiments of Kyushu University and was carried out according to the Guidelines for Animal Experiments of Kyushu University and the Japanese Government.

Animals

AKR female mice (H-2k, aged 9 to 11 weeks) were used as heart donors, and C3H/He (H-2k) female mice (allograft transplantation) and AKR female mice (isograft transplantation) of the same age were used as recipients. A total of 258 mice (Japan SLC Inc, Tokyo, Japan, or Seac Yoshitomi, Tokyo, Japan) were used in this study. The animals were housed to have free access to food and drink and were maintained at 23±2°C with 12-hour light and dark cycle.

Cardiac Transplantation and Drug Administrations

Heterotopic cervical cardiac transplantation was performed by the standard technique.11 A day before cardiac transplantation, recipients...
were randomized into the following 3 groups and pharmacological treatment with fasudil was started: recipients transplanted with allografts with (fasudil) group or without (control) group) oral treatment with fasudil (Asahi Kasei Corp, Tokyo, Japan; 10 and 30 mg/kg per day in drinking water) and recipients with isografts without any drug (isograft group). In a preliminary study, we checked the volume of daily water intake of recipient animals for 4 weeks after the transplantation. The recipients were able to freely access the water in which fasudil was dissolved. The amount of fasudil with which the animals were treated was calculated with recipient weight and drinking water volume. We have previously confirmed that fasudil is metabolized to hydroxyfasudil, a specific inhibitor of Rho-kinase, after oral administration.12 We have previously confirmed that the inhibitory effect of hydroxyfasudil on Rho-kinase is 100 times higher than for protein kinase C (PKC) and 100 times higher than for myosin light-chain kinase.12 Furthermore, the inhibitory effect of hydroxyfasudil on 16 kinases, including Rho-kinase, has recently been examined. Among the kinases tested, hydroxyfasudil at 10 μM showed more than 50% inhibition only for Rho-kinase (97.6%).13 Thus, we consider that hydroxyfasudil is a reasonably selective inhibitor for Rho-kinase in the present study. Plasma concentrations of fasudil and hydroxyfasudil were measured by high-performance liquid chromatography at 4 weeks after the transplantation.14

Adenovirus-Mediated In Vivo Gene Transfer
Adenovirus vectors encoding a mutant (NK1036→TT) Rho-binding (RB) domain of Rho-kinase plus a pleckstrin homology domain (RB/PH[TT]; 2.2×10^9 pfu/mL in 0.15 mL),15 which is a dominant-negative form of Rho-kinase (DN-Rho-kinase), and those with LacZ (2.3×10^10 pfu/mL, in 0.15 mL as a control) were transfected to negative form of Rho-kinase (DN-Rho-kinase), and those with LacZ histologically.

Cardiac grafts were homogenized in 0.8 mL of Isogen (Wako Pure Chemical Ind, Osaka, Japan), and total RNA was extracted according to the manufacturer’s protocol. The RNAse protection assay was performed using a multipurpose assay system (PharMingen, San Diego, Calif) for cytokines, chemokines, and adhesion molecules, including tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), transforming growth factor-β1 (TGF-β1), interleukin-6 (IL-6), macrophage migration inhibitory factor (MIF), monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin. Isotope-labeled hybridization reactions were electrophoresed on 5% acrylamide gel, and this gel was exposed to scientific imaging film (Kodak Inc, Rochester, NY). Areas of the respective transcript bands were measured and were normalized against that of GAPDH.

Histology and Morphology
All grafts were perfused with sodium nitroprusside (10^-3 mol/L) as a vasodilator and fixed with paraldehyde and formaldehyde under 180 cm H2O pressure before embedding in paraffin. The grafts were cut transversely into 3 blocks, fixed in 4% phosphate-buffered paraldehyde, and embedded in paraffin. Three sections (5 μm thick) were made from each block and stained with Masson’s trichrome. The intima, media, and perivascular fibrosis areas were measured at a magnification of ×200 (BX50F-3, Olympus Optical Co, Tokyo, Japan). The ratio of the intimal area to total vascular area and that of the perivascular fibrosis area to total vascular area were calculated.

Western Blot Analysis
Four weeks after the transplantation, cardiac grafts were isolated and total protein was extracted from each graft. The extent of phosphorylation of ezrin, radixin, and moesin (ERM), the substrates of Rho-kinase, has recently been examined. Among the kinases tested, hydroxyfasudil at 10 μM showed more than 50% inhibition only for Rho-kinase (97.6%).13 Thus, we consider that hydroxyfasudil is a reasonably selective inhibitor for Rho-kinase in the present study. Plasma concentrations of fasudil and hydroxyfasudil were measured by high-performance liquid chromatography at 4 weeks after the transplantation.14

Isolation of RNA and Ribonuclease Protection Assay
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Immunohistochemistry
Four weeks after the transplantation, cardiac grafts were cut horizontally into 4 blocks, embedded in OTC compound (Sakura Finetechical Co, Tokyo, Japan) and kept at –80°C until staining. Nine slices (3 slices from 3 blocks from the apex) were made for immunostaining with a kit (Histofine SAB-PO kit, Nichirei Co, Tokyo, Japan) for macrophages (MOMA-2) and CD4- and CD8-positive T cells, as previously described.19 Three fields where a coronary artery was recognized in the center were selected from each animal to count the number of macrophages and calculate a percent-positive area in a blind manner at a magnification of ×200.

Statistical Analysis
All results are expressed as the mean±SEM. Data were analyzed either by unpaired t test or by ANOVA followed by Fisher’s post hoc test for multiple comparisons. Values of P<0.05 were considered to be statistically significant.

Results
Cardiac Allograft Vasculopathy
Mice treated with fasudil were well tolerated and showed no side effects, such as weight loss, hair loss, or diarrhea. A total of 2369 coronary arteries were evaluated by computer-assisted analysis in terms of the severity of CAV. Four weeks after the cardiac transplantation from AKR to C3H/He mice, neointima formation (evaluated by intima/vascular area ratio) and perivascular fibrosis of coronary arteries were markedly enhanced in the control allograft group compared with the isograft group (Figures 1 and 2). By contrast, coronary veins
were resistant to those changes (Figure 1E). In the isograft group, perivascular fibrosis was also developed probably due to a reperfusion injury alone (Figure 2B). Both neointima formation and perivascular fibrosis of the allografts were dose-dependently attenuated in the fasudil groups (Figures 1 and 2). The high dose of fasudil inhibited the perivascular fibrosis to the level seen in the native hearts (Figure 2B). The medial area of the coronary artery was reduced only in the high-dose fasudil group compared with the control group (Figure 2C). The medial area in the high-dose fasudil group (0.31 ± 0.01, n = 9) was equal to that seen in the native hearts (0.36 ± 0.01, n = 10). At 4 weeks after the treatment with fasudil, plasma concentrations of hydroxyfasudil (ng/mL) increased from 0 (control animals) to 5.53 ± 2.08 and 37.24 ± 19.12 in the low-dose (n = 6) and the high-dose (n = 5) fasudil groups, respectively, specific therapeutic ranges of the Rho-kinase inhibitor.12 By contrast, fasudil was not detected in any groups.

Rho-Kinase Activity
The extent of ERM phosphorylation, as normalized by that of total actin, was significantly enhanced in the control allograft group compared with the isograft group (Figure 3). The long-term treatment with fasudil dose-dependently suppressed the increase in Rho-kinase activity in the allograft group (Figure 3). The total amount of ERM did not change among the 4 groups (Figure 3). The actin density was significantly less in the allograft group than any other groups because of the abundant ECM in the equal amount of the sample.

In Vivo Gene Transfer of DN-Rho-Kinase
To confirm the specificity of the inhibitory effect of hydroxyfasudil on CAV, adenovirus-mediated gene transfer of DN-Rho-kinase was performed while LacZ transfection was used as a control. X-gal staining demonstrated that LacZ was expressed widely in the cardiac grafts (Figure 4A). Histological analysis showed that the gene transfer of DN-Rho-kinase suppressed both intimal thickening (evaluated by intima/vascular area ratio) and perivascular fibrosis compared with that of LacZ (Figures 4B and 4C). In this experiment, since the extent of myocardial fibrosis was too high to identify some small blood vessels, we examined only relatively larger arteries where intimal thickening was prominent while perivascular fibrosis was less prominent. Thus, compared with the results obtained in the fasudil protocol (Figure 2), the extent of intimal thickening was relatively greater while that of perivascular fibrosis was relatively smaller (Figure 4).

Expressions of Inflammatory Molecules
RNase protection assay demonstrated that the expression of MIF, IFN-γ, and TGF-β1 in the allografts was significantly upregulated in the control group and was dose-dependently inhibited in the fasudil group (Figure 5). Only the expression of MIF was inhibited by a low dose of fasudil (Figure 5A), which also was effective to suppress the development of CAV (Figure 2A). The expression of TNF-α, MCP-1, VCAM-1, ICAM-1, and E-selectin in the allografts also was upregulated in the control group but was not significantly suppressed in the fasudil group (data not shown). IL-6 was not detected in any group examined.
Inflammatory Cell Infiltration

Immunostaining demonstrated that a number of infiltrating macrophages (MOMA-2) and CD4- or CD8-positive T cells and their percent-positive area were minimal in the isografts (Figures 6 and 7). Both of them were significantly enhanced in the allograft group compared with the isograft group, which was significantly suppressed with the fasudil treatment (Figures 6 and 7).

Discussion

This study has revealed three novel findings as follows. First, the long-term treatment with fasudil dose-dependently suppressed the development of CAV, the effect of which was associated with the decrease in the Rho-kinase activity. Second, the beneficial effect of hydroxyfasudil was qualitatively mimicked by the in vivo gene transfer of DN-Rho-kinase. Third, the expression of several cytokines was upregulated in the allografts and was suppressed by the fasudil treatment, with the significant inhibitory effect noted for MIF, IFN-γ, and TGF-β1. These results suggest that Rho-kinase is substantially involved in the pathogenesis of CAV, implicating a potential usefulness of Rho-kinase inhibitors to prevent the disorder.

An effective strategy to suppress CAV has yet to be developed. In previous studies, angiotensin-converting enzyme inhibitor, angiotensin II receptor antagonist, and 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors were shown to cause a 30% to 45% inhibition of the disorder. An inhibitory effect of hydroxyfasudil (≈90%), at safe doses, is more prominent than that of any of those drugs tested before, such as CGP53716, an inhibitor of the platelet-derived growth factor tyrosine kinase. In the present study, the plasma level of hydroxyfasudil was 37.24 ± 19.12 ng/mL (0.11 ± 0.06 μmol/L), which is within its clinical therapeutic level, suggesting that the oral treatment with fasudil is safe for both mice and humans. Thus, Rho-kinase could be regarded as an important molecular target for the prevention of CAV.

Mouse Model of CAV

Although several studies were performed to elucidate the mechanisms of CAV, the pathogenesis of the disorder still remains unclear. Recently, a novel murine model of long-
term CAV was developed using an H-2 identical combination, AKR (H-2^k) to C3H (H-2^k). In this combination, because of a mismatch of minor antigens, the process of CAV is initiated as early as 2 weeks after grafting and is further developed at 4 weeks. Since the coronary vascular lesions in this model have many similarities to those in humans, the model has been used to examine the pathogenesis of CAV.

**Inhibitory Effects of Hydroxyfasudil on CAV**

In the present study, intimal thickening of coronary arteries was suppressed by the long-term treatment with fasudil. To confirm the inhibitory effect of hydroxyfasudil on Rho-kinase, we also examined the effects of in vivo gene transfer of dominant-negative Rho-kinase. Compared with the results obtained in the drug protocol (Figure 2), the extent of intimal thickening was relatively greater while that of perivascular fibrosis was relatively smaller (Figure 4). This observation traces its source to the use of adenovirus as a vector. Since the extent of myocardial fibrosis was too high to identify some small blood vessels, we examined only relatively larger arteries where intimal thickening was prominent while perivascular fibrosis was less prominent.

In the present study, perivascular fibrosis in the allografts was enhanced in the control group compared with the isograft group and was markedly inhibited by long-term hydroxyfasudil. The extent of perivascular fibrosis in the high-dose fasudil group was equivalent to that in native hearts, a finding consistent with our previous study. Since perivascular fibrosis in cardiac allografts is caused by both immune response and a reperfusion injury (as seen in isografts), hydroxyfasudil appears to inhibit both processes.

Regarding the medial thickening of coronary arteries, the value in the high-dose fasudil group was equal to that in native hearts. In the present study, a high dose of fasudil did not cause medial changes, which is in contrast to the previous report. The discrepancy is probably due to some differences in experimental conditions between the present and the previous study. First, we examined mouse coronary arteries whereas rabbit carotid arteries were examined in the previous study. Second, we used a transplant model whereas a balloon injury model was used in the previous study.
Mechanisms of Action of Hydroxyfasudil

Several mechanisms could be involved in the inhibitory effects of hydroxyfasudil. First, hydroxyfasudil could facilitate apoptotic cell death in the neonintima as does Y-27632, another specific Rho-kinase inhibitor.\textsuperscript{30,31} In addition, cytoplasmic translocation of ERM may be involved in an early phase of apoptosis.\textsuperscript{32} Second, hydroxyfasudil could inhibit cell migration.\textsuperscript{17,30,33} Third, hydroxyfasudil could suppress VSMC proliferation.\textsuperscript{6}

Rho-kinase plays an important role in the pathogenesis of arteriosclerosis in vivo.\textsuperscript{6} We have recently demonstrated that Rho-kinase–mediated phosphorylation of ERM and adducin is increased at arteriosclerotic coronary lesions in pigs and that long-term blockade of Rho-kinase by either hydroxyfasudil\textsuperscript{14} or in vivo gene transfer of DN-Rho-kinase\textsuperscript{15} induces a regression of the coronary lesions in vivo. Regarding the measurement of Rho-kinase activity in our study, we consider that the activity is significantly increased in our mouse model of CAV for the following reasons. First, we consider that a 2-fold increase in Rho-kinase activity is significant because this measurement only represents the whole level of Rho-kinase activity of the heart, and some population of vascular wall cells (eg, VSMCs and inflammatory cells) may have much higher activity of Rho-kinase. Second, the increased activity of Rho-kinase in CAV was normalized not only by pharmacological blockade of Rho-kinase with hydroxyfasudil but also by in vivo gene transfer of DN-Rho-kinase. Third, as shown in Figure 3, the total ERM level was unchanged in the present study.

Antiinflammatory Effects of Hydroxyfasudil

In the present study, hydroxyfasudil dose-dependently suppressed upregulated MIF, IFN-\gamma, and TGF-\beta1 expression in allografts (Figure 5). We consider that hydroxyfasudil inhibits the inflammatory responses mediated by those cytokines and resultant formation of CAV. We have no definite explanations why some cytokines were selectively upregulated in our CAV model in a Rho-kinase–dependent manner. One possible explanation is that the contribution of Rho-kinase to cytokine expression may be variable depending on the condition of CAV and/or animals used. It has been reported that Rho-kinase regulates gene expression of plasmogen activator inhibitor–1 (PAI-1) but not extracellular signal–regulated protein kinase.\textsuperscript{35}

MIF may be involved in the pathogenesis of graft rejection\textsuperscript{36} and atherosclerosis.\textsuperscript{37} Although we did not examine the molecular mechanism for the connection between Rho-kinase and MIF in this study, we have recently demonstrated that Rho-kinase is substantially involved in the upregulation of inflammatory molecules, such as PAI-1\textsuperscript{38} and the downregulation of eNOS.\textsuperscript{10} It has been reported that MIF upregulates the expression of ICAM-1 on endothelial cells while it decreases redox– or stress-induced apoptosis.\textsuperscript{37} It is important to note that a low dose of fasudil, which suppressed the development of CAV, inhibited the expression of MIF alone. Thus, it is conceivable that MIF plays an important role in the pathogenesis of CAV in the present model. IFN-\gamma also may be involved in the progression of CAV. The development of CAV is suppressed in the grafts from IFN-\gamma–deficient mice, suggesting an involvement of the cytokine in the pathogenesis of the disorder.\textsuperscript{38,39} TGF-\beta1 is known to increase fibroblast and type I collagen expression by fibroblasts.\textsuperscript{40} In addition, an increased expression of fibroectin and laminin in the early posttransplantation period precedes cellular infiltration.\textsuperscript{41,42} Thus, it is also conceivable that Rho-kinase–mediated upregulation of TGF-\beta1 is involved in the pathogenesis of CAV. In the present study, the expression of TNF-\alpha, MCP-1, VCAM-1, ICAM-1, and E-selectin in the allografts was also upregulated in the control group but was not significantly suppressed in the fasudil group. This may suggest that hydroxyfasudil does not directly suppress CAV but rather downregulates inflammation across the whole graft, including the vasculature.

Limitations of the Study

Several limitations of the present study should be mentioned. First, the present model may not completely represent clinical cardiac transplantation partly because heterotopic cardiac transplantation was performed in this study and partly because only minor tissue mismatches are carried in the allografts. However, as discussed above, the present model is useful for examining the mechanisms of CAV.\textsuperscript{27,28} Second, the whole heart was used for molecular analyses since it is difficult to isolate a sufficient amount of coronary arteries from the mouse heart. This means that the relevant findings may not specifically relate to the pathogenesis of CAV. Third, the potential effects of immunosuppressive agents on the development of CAV were not examined in the present study.

In summary, the present study demonstrates that hydroxyfasudil, a metabolite of fasudil, may act on Rho-kinase and possibly may have other antiinflammatory properties. The suppression of CAV by hydroxyfasudil in mice suggests that Rho-kinase is an important therapeutic target for the prevention of the disorder.

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References

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