Long-term inhibition of Rho kinase suppresses intimal thickening in autologous vein grafts in rabbits

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Background: **Rho kinase plays an important role in vascular smooth muscle cell (VSMC) contraction and other cellular functions, such as proliferation, migration, and apoptosis. Recent studies have demonstrated that long-term inhibition of Rho kinase suppresses coronary artery spasm and vascular lesion formation after arterial injury. In the cardiovascular surgery field, intimal thickening in vein grafts is the major cause of late graft failure, for which no effective treatment has yet been developed. In this study, we examined whether long-term inhibition of Rho kinase suppresses intimal thickening in autologous vein grafts in rabbits.**

Methods: **Male rabbits were randomly divided into two groups and received normal chow (control group) or a special chow containing 0.09% fasudil (fasudil group). After oral administration, fasudil is metabolized to a specific Rho kinase inhibitor, hydroxyfasudil. Each group underwent reversed autologous vein graft surgery with the internal jugular vein into the left common carotid artery. At 1, 2, and 4 weeks after the operation, we examined the extent of intimal thickening of the graft and VSMC proliferation and apoptosis.**

Results: **The intimal thickening was significantly suppressed in the fasudil group compared with the control group at 2 and 4 weeks after the operation. In the fasudil group, VSMC proliferation was suppressed at 1 and 2 weeks after the operation, whereas VSMC apoptosis was enhanced at 2 weeks after the procedure.**

Conclusions: **These results indicate that Rho kinase is substantially involved in the pathogenesis of intimal thickening of vein grafts and that it is an important therapeutic target for the prevention of graft failure. (J Vasc Surg 2006;43: 1249-56.)**

For vascular occlusive diseases, an autologous vein graft is the most suitable conduit for arterial reconstruction; however, late graft failure remains a serious problem that limits the long-term efficacy of the procedure.[1] Among the possible mechanisms involved, intimal thickening seems to be the major cause of the disorder.[2,3] It is characterized by enhanced migration and proliferation of vascular smooth muscle cells (VSMCs) from the media into the intima after transplantation into the arterial circulation.[4,5] Several experimental and clinical studies have attempted to reduce the intimal thickening of the graft. However, no effective pharmacologic therapy has yet been developed for human use.[4,5]

Rho kinase, a target protein of the small guanosine triphosphatase Rho, regulates vascular tone through a Ca^{2+} sensitization mechanism and plays an important role in VSMC contraction.[6,7] Phosphorylation of the myosin light chain (MLC) is one of the essential steps for VSMC contraction, the level of which is regulated by MLC kinase and MLC phosphatase.[6,7] Recent studies have demonstrated that Rho regulates MLC phosphorylation through Rho kinase and MLC phosphatase.[8,9] The Rho/Rho kinase pathway also plays an important role in other cellular functions, such as actin cytoskeleton organization, adhesion and migration, cytokinesis, proliferation, and apoptosis.[10-13] We have recently demonstrated that long-term inhibition of Rho kinase by either a Rho kinase inhibitor or in vivo gene transfer of dominant-negative Rho kinase suppresses coronary artery spasm and vascular lesion formation in various animal models.[14-19] Thus, this study was designed to examine whether long-term inhibition of Rho kinase sup-

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presses intimal thickening of vein grafts in rabbits and, if so, to elucidate the mechanisms involved.

METHODS

This study was reviewed by the Committee on Ethics on Animal Experiments of Kyushu University and was performed under the Guidelines for Animal Experiments of Kyushu University and the law (No. 105) and the notification (No. 6) of the Japanese Government.

Autologous vein graft surgery. A total of 70 male Japanese White rabbits weighing 2.5 to 3.0 kg were randomly divided into 2 groups: (1) a control group that received normal chow and (2) a fasudil group that received a special chow containing 0.09% fasudil 30 mg/kg. In a previous study, long-term administration of fasudil (30 mg/kg) was effective for suppressing arterial neointimal formation in the porcine model[20] and for suppressing cardiovascular hypertrophy in the rat model.[21] Therefore, we used this dose in the current study. Fasudil is metabolized to a selective inhibitor for Rho kinase,[21] hydroxyfasudil, after oral administration.[16,17] The fasudil treatment was started 3 days before the operation and was continued for 4 weeks, until the end of the experiment. Anesthesia was induced with xylazine (10 mg/kg subcutaneously) and ketamine hydrochloride (25 mg/kg intramuscularly) and was maintained with pentobarbital sodium (30 mg/kg intravenously). Under sterile conditions, the left internal jugular vein and the left common carotid artery were exposed, and the branches of the jugular vein were ligated.[22] The vein was then excised and was kept moistened in heparinized (5 U/mL) saline solution. Heparin (1000 U per body) was administered intravenously. The left carotid artery was clamped at the proximal and distal ends. A proximal longitudinal arteriotomy was made, and the vein was reversed and anastomosed to the artery in an end-to-side manner.[22] Distal anastomosis was performed in a similar manner, and then the carotid artery was divided. The wound was closed in layers.

Blood sample analysis. Blood was obtained from the middle ear artery at the time of operation and at 1, 2, and 4 weeks after the operation. In addition, plasma concentrations of hydroxyfasudil were assayed by high-performance liquid chromatography.

Blood pressure measurement. At the operation and 4 weeks after the operation, blood pressure was measured through a cannula placed in the middle ear artery by using a pressure transducer connected to a polygraph monitoring system. All measurements were made 5 minutes after the induction of anesthesia, when all hemodynamic variables were stabilized.

Harvest of implanted vein grafts. At 1, 2, and 4 weeks after the operation, the animals in both groups were killed with a lethal dose of pentobarbital (n = 7 in each group). The vein grafts were exposed and removed and were perfusion-fixed in situ at 100 cm H_2O for 15 minutes. The perfused vein graft, including the anastomosis, was immersed in neutralized 10% formaldehyde overnight at room temperature.

MLC phosphorylation. To examine Rho kinase activity, we measured the extent of phosphorylated MLC in vein grafts at 4 weeks after the operation with serotonin at a concentration of 10^{-6} mol/L. The extent of MLC phosphorylation in the grafts was determined by using the urea/glycerol gel method, followed by immunoblot detection with a specific mouse monoclonal anti-MLC antibody, as previously described.[16,17,23] Both unphosphorylated and phosphorylated forms of the 20-kd MLC were detected by the specific antibody (×200 dilution) and a secondary antibody (×1000 dilution). The region containing MLC was visualized with an ECL-Plus Western blotting system (Amersham, Buckinghamshire, UK). After the image of the radiograph film was obtained with a charge-coupled device camera (Affo, Tokyo, Japan), the density of unphosphorylated and phosphorylated MLCs was determined by an NIH Image analyzer (version 1.61). The percentage of the phosphorylated form in the total MLC was determined to express the extent of MLC phosphorylation.

Organ chamber experiment. Four weeks after the operation, VSMC contractile responses were examined in vitro. The vein grafts from the control and the fasudil group (n = 4 each) were cut into rings 3 to 4 mm in length. Because we have repeatedly confirmed that the endothelium has no inhibitory effect on the contractions of normal veins or vein grafts,[24] we used rings with endothelium throughout the experiment. The rings were suspended by hooks in an organ bath that contained oxygenated Krebs solution of the following composition (mmol/L): NaCl 121.3, KCl 4.7, $NaHCO_3$ 24.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, and D-glucose 5.77, bubbled with 95% oxygen and 5% carbon dioxide and maintained at 37 °C.[19] The hooks were connected to a force transducer (Nihon Kohden, Tokyo, Japan), and the data were stored in a computer using MacLab. After a 40-minute equilibration period, the rings were stimulated with 60 mmol/L KCl every 30 minutes, and the resting tension was increased in a stepwise manner to achieve a maximal force development. The rings were then exposed to increasing concentrations of KCl (5-100 mmol/L). After washout, the vasoconstrictor responses to increasing concentrations of serotonin (10^{-9} to 10^{-5} mol/L) were examined in the presence and absence of hydroxyfasudil (10^{-5} mol/L). The responses were expressed as a percentage of the contractions to 60 mmol/L KCl that caused maximal contractions.[25]

Intimal thickening. The sections were stained with hematoxylin and eosin and Elastica–van Gieson stainings. Four sections were obtained from each vein graft. The extent of maximal intimal thickness was measured by Macscope (Mitani Co, Fukai, Japan) at eight random points in each section.[22] The quantitative analysis was performed in four independent sections. The average of the eight points was considered to represent the intimal thickening of the section, and the average of the four sections was considered to represent that of the graft. The intimal area was also calculated in the same manner. For the examination of constructive remodeling, we measured the

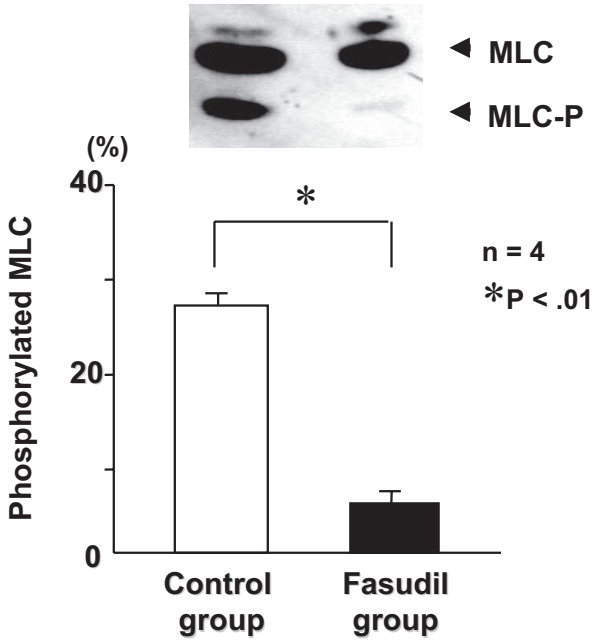

Fig 1. Suppression of myosin light chain *(MLC)* phosphorylation *(MLC-P)* by fasudil. The extent of MLC phosphorylation was significantly inhibited in the fasudil group compared with the control group. Results are expressed as the mean ± SEM.

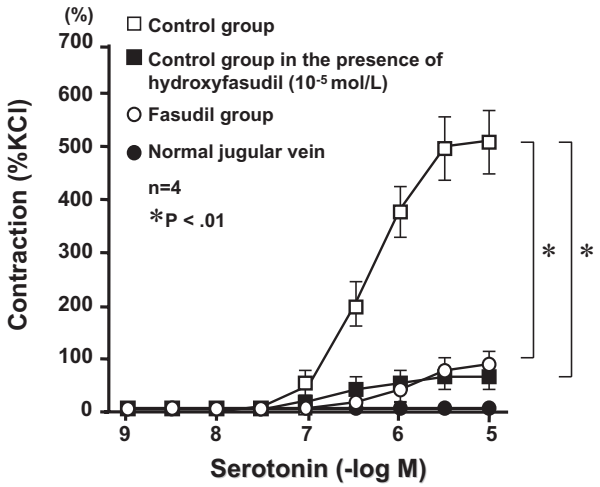

Fig 2. Suppression of vascular smooth muscle cell (VSMC) hypercontraction of vein grafts by fasudil. In the control group, serotonin caused VSMC hypercontractions as compared with the normal jugular vein, and these hypercontractions were significantly inhibited by acute administration of fasudil to the level seen in the fasudil group. Results are expressed as the mean ± SEM.

Table. Intimal thickening (μm) of vein grafts

Time (wk)	*Control group (n = 7)*	*Fasudil group (n = 7)*
1	26.19 ± 0.99 (20.41-31.22)	22.63 ± 0.46 (18.93-27.50)
2	54.58 ± 3.27 (27.05-82.50)	34.86 ± 2.96* (21.63-62.79)
4	74.37 ± 2.99 (48.78-111.95)	47.64 ± 2.84* (23.63-83.48)

Data are mean ± SEM (range).
*$P < .01$ vs control group.

inside diameter of the internal elastic lamina at 4 weeks after the operation. All analyses were performed in a blinded manner.

Immunohistochemical stainings. Proliferating cell nuclear antigen (PCNA) staining was performed as follows. Briefly, formalin-fixed, paraffin-embedded tissue sections were cut into 3-μm sections, mounted on silanated slides, deparaffinized in xylene, and rehydrated in a graded series of ethanol. Endogenous peroxidase activity was blocked by using methanol containing 0.3% H_2O_2. To avoid nonspecific binding, the slides were preincubated with 3% skim milk for 30 minutes. Sections were incubated with primary monoclonal antibody against PCNA (DAKO, Kyoto, Japan) and refrigerated overnight at 4 °C. The primary antibody was visualized with a streptavidin-biotin-peroxidase kit (Nichirei, Tokyo, Japan) with diaminobenzidine tetrahydrochloride as a chromogen. These sections were then lightly counterstained by hematoxylin.

For detection of apoptotic cells, we performed terminal transferase-mediated deoxyuride-5′-triphosphate nick-end labeling (TUNEL) staining (Takara In Situ Apoptosis Detection Kit; Takara Co, Ohtsu, Japan) in accordance with the manufacturer's protocol. The counterstaining was performed with methyl green. VSMCs and macrophages were detected by monoclonal mouse anti-human α-smooth muscle actin (DAKO) and monoclonal mouse anti-rabbit macrophage 11 (DAKO), respectively.

The number of PCNA- and TUNEL-positive VSMCs was counted in the intima at a magnification of ×400 in a blinded manner, and the ratio of the number of PCNA- and TUNEL-positive cells to that of total VSMCs was obtained. The quantitative analysis was performed in eight independent sections. The average of the eight values was considered to be the ratio of positive cells in one section, and the average of the four values was considered to represent that of one vein graft.

Statistical analysis. All results are expressed as the mean ± SEM. The data were analyzed by using one-way analysis of variance followed by the Scheffé post hoc test. A value of $P < .05$ was considered to be statistically significant.

RESULTS

Animals and graft patency. Eight rabbits were used for the examination of MLC phosphorylation. Sixteen rabbits were used for the organ chamber experiment. Forty-two rabbits were used for the analysis of intimal thickening and immunohistochemical stainings. Four rabbits died during the induction of intravenous anesthesia (pentobarbital sodium). All vein grafts were patent until the harvest.

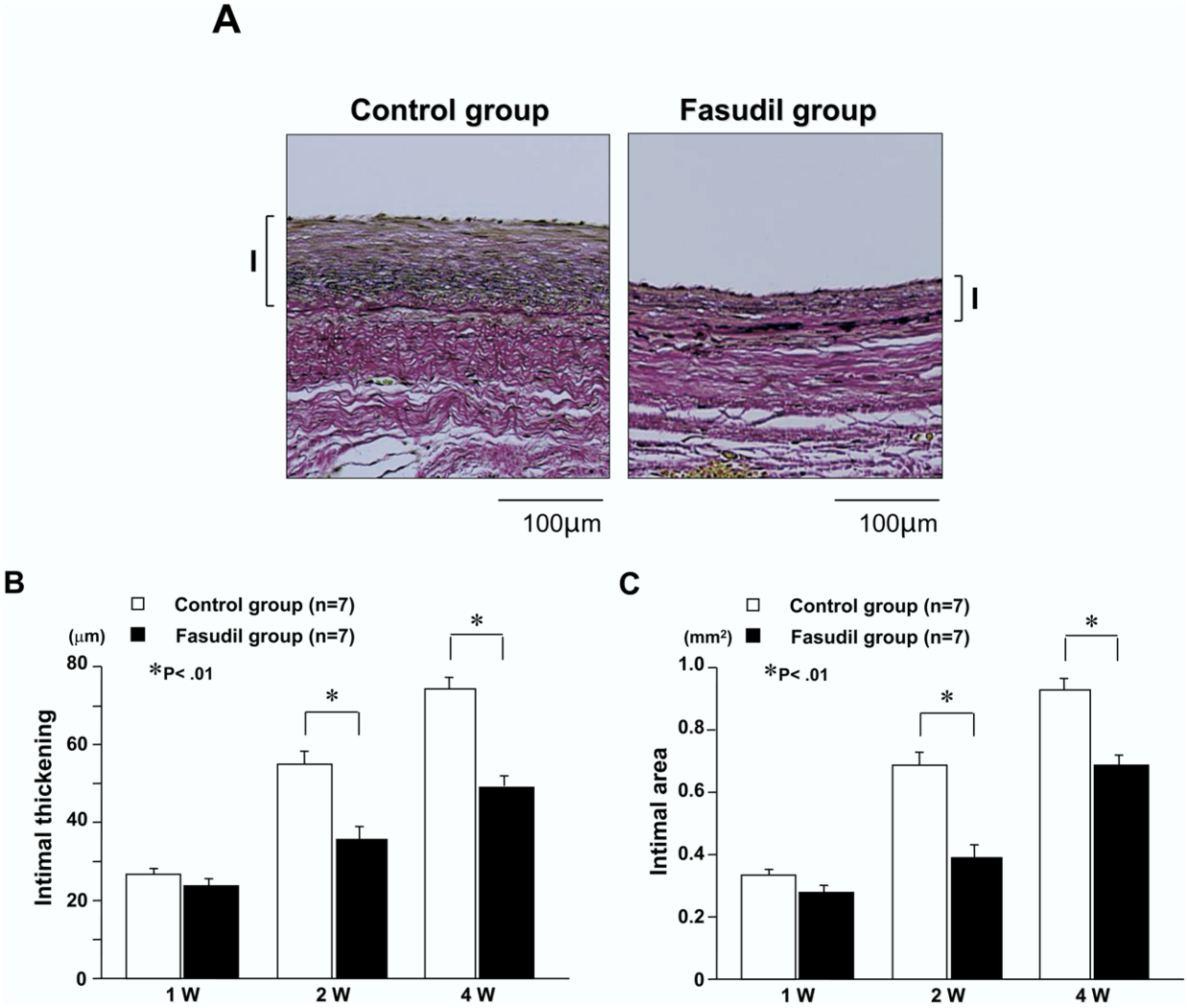

Fig 3. Suppression of intimal thickening of the vein graft by fasudil. (**A**) Representative photographs of the mid portion of an autologous vein graft from the control and the fasudil groups 4 weeks after the operation (Elastica–van Gieson stain; original magnification, ×200). Intimal thickening (as shown by *I*) was suppressed in the fasudil group compared with the control group. (**B**) Quantitative analysis of the intimal thickness of vein grafts. The intimal thickening was significantly suppressed in the fasudil group at 2 and 4 weeks *(W)* after the operation. (**C**) Quantitative analysis of the intimal area of vein grafts. The intimal area was significantly suppressed in the fasudil group at 2 and 4 weeks *(W)* after the operation. Results are expressed as the mean ± SEM.

Systemic measurements. There was no significant difference in body weight between the groups (data not shown). Systolic blood pressure was also similar between the control and the fasudil groups (92 ± 5 mm Hg and 89 ± 2 mm Hg at the operation and 87 ± 2 mm Hg and 87 ± 1 mm Hg at 4 weeks after the operation, respectively; n = 7 in each group). The plasma concentrations of hydroxyfasudil were 0 ng/mL in the control group and 251 ± 58 ng/mL, 197 ± 70 ng/mL, and 270 ± 66 ng/mL in the fasudil group at 1, 2, and 4 weeks after the operation, respectively (n = 4 each). These concentrations of hydroxyfasudil were within the specific therapeutic range of the Rho kinase inhibitor.[16,17]

Suppression of MLC phosphorylation by fasudil. The extent of MLC phosphorylation (the phosphorylated form in the percentage of total MLC) at 4 weeks after the operation was significantly suppressed in the fasudil group compared with the control group (Fig 1).

Suppression of VSMC hypercontraction by fasudil. There was no significant difference in the KCl-induced maximal contraction between the control (3.47 ± 0.42 g) and the fasudil (3.87 ± 0.41 g) groups. Serotonin caused hypercontractions of vein grafts in the control group, which were markedly inhibited by acute administration of hydroxyfasudil to the level seen in the fasudil group (Fig 2). In contrast, serotonin caused no contraction of the normal jugular vein.

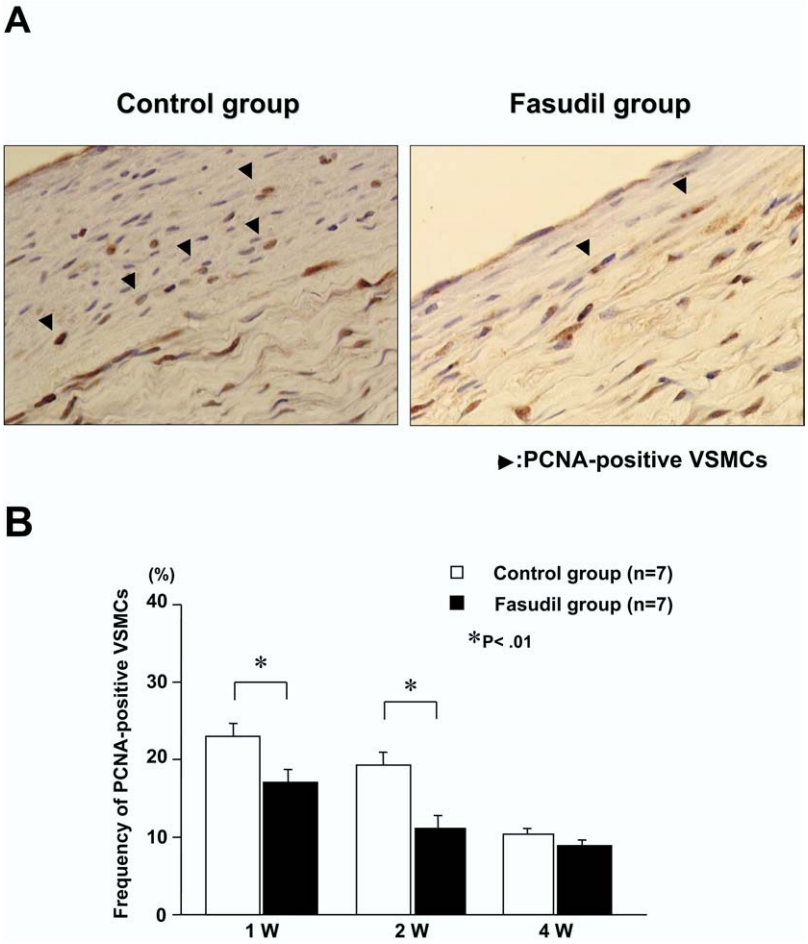

Fig 4. Vascular smooth muscle cell *(VSMC)* proliferation in vein grafts. (**A**) Microscopic findings with proliferating cell nuclear antigen *(PCNA)* staining of the mid portion of an autologous vein graft from the control and fasudil groups 2 weeks after the operation. *Arrowheads* show PCNA-positive VSMCs (original magnification, ×400). (**B**) Quantitative analysis of the frequency of PCNA-positive cells. The frequency was significantly suppressed in the fasudil group at 1 and 2 weeks *(W)* after the operation. Results are expressed as the mean ± SEM.

Suppression of intimal thickening by fasudil. In the control group, intimal thickening, as evaluated by either maximal intimal thickness or intimal area, progressively developed and was significantly suppressed in the fasudil group at 2 and 4 weeks after the operation (Table; Fig 3). Four weeks after the operation, the inside diameter of the internal elastic lamina was 4779.8 ± 339.5 μm in the control group and 3892.9 ± 314.0 μm in the fasudil group (not significantly different).

Suppression of VSMC proliferation by fasudil. The number of PCNA-positive cells, a marker of VSMC proliferation,[22,26] was significantly reduced in the fasudil group compared with the control group at 1 and 2 weeks after the operation (Fig 4).

Enhancement of VSMC apoptosis by fasudil. VSMC apoptosis was evaluated by TUNEL staining.[26,27] Two weeks after the operation, the number of TUNEL-positive VSMCs was significantly higher in the fasudil group compared with the control group (Fig 5).

No influence of fasudil on macrophage infiltration. The number of macrophages detected by anti-rabbit macrophage 11 staining (number per ×400 visual field) was low throughout the experimental period in the control and the fasudil groups: 0.7 ± 0.2 and 0.9 ± 0.3 at 1 week, 0.9 ± 0.2 and 0.7 ± 0.2 at 2 weeks, and 0.4 ± 0.1 and 0.5 ± 0.1 at 4 weeks after the operation, respectively (no statistical differences were found).

DISCUSSION

The novel findings of this study were that (1) long-term inhibition of Rho kinase by fasudil suppressed the development of intimal thickening of vein grafts and (2) suppression of VSMC serotonin-induced hypercontraction and proliferation and enhancement of VSMC apoptosis were involved in the inhibitory effects of fasudil. To the best of our knowledge, this is the first report that demonstrates the

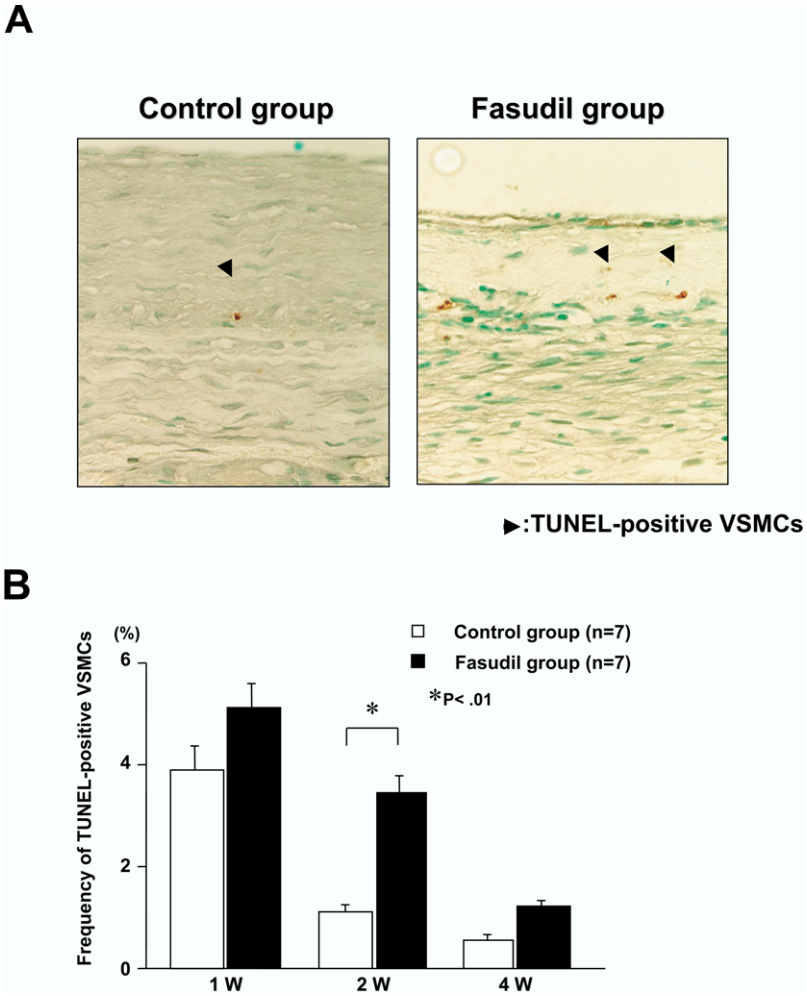

Fig 5. Terminal transferase-mediated deoxyuride-5′-triphosphate nick-end labeling *(TUNEL)*-positive vascular smooth muscle cells *(VSMCs)* in thickened intima. (**A**) Microscopic findings with TUNEL staining of the mid portion of autogenous vein grafts from the control and fasudil groups 2 weeks after the operation. *Arrowheads* show TUNEL-positive cells (original magnification, ×400). (**B**) Quantitative analysis of the frequency of TUNEL-positive cells. The frequency was significantly higher in the fasudil group compared with the control group 2 weeks *(W)* after the operation. Results are expressed as mean ± SEM.

usefulness of Rho kinase inhibitors for the prevention of vein graft disease.

Hydroxyfasudil, which is a metabolite of fasudil after oral administration, is a specific inhibitor of Rho kinase.[16,17] Fasudil is used for the treatment of cerebral vasospasm after subarachnoid hemorrhage. In this study, fasudil did not influence systemic blood pressure, body weight, or survival until 4 weeks after the operation. In humans, oral administration of fasudil is well tolerated, without any serious adverse reactions.[18,28]

Rho kinase enhances the Ca^{2+} sensitivity of VSMCs through inhibition of MLC phosphatase, which results in enhanced MLC phosphorylation.[6,7] In this study, MLC phosphorylation was significantly inhibited in the fasudil group, thus demonstrating an inhibition of Rho kinase by long-term oral treatment with fasudil. We have recently demonstrated that Rho kinase is substantially involved in the pathogenesis of hypercontraction of arteriosclerotic arteries and of coronary artery spasm both in animals and humans.[14-19,28] In this study, serotonin-induced contractions of vein grafts were enhanced, which is consistent with our previous report[25]; these were markedly suppressed in the fasudil group.

VSMC serotonin-induced hypercontraction and proliferation seem to be closely coupled in terms of an involvement of Rho kinase, because we have recently observed that long-term inhibition of Rho kinase, either by in vivo gene transfer of dominant-negative Rho kinase or by long-term treatment with fasudil, causes a regression of both vascular vasospastic activity and arteriosclerosis.[14-19] A similar result has also been reported in a clinical study with patients with vasospastic angina, in which coronary vasospastic activity

and arteriosclerotic lesions at the spastic segment are closely coupled.[29]

Intimal thickening of autologous vein grafts is the major cause of late graft failure.[2,3] The intimal thickening is characterized by enhanced proliferation and migration of VSMCs from the media into the intima.[4,5] We have previously reported that intimal thickening was observed as early as 1 week after the operation and thereafter progressively increased,[30] whereas another study showed that VSMC proliferation in the intima peaked from 1 to 2 weeks after the operation.[31,32] We also reported that intimal thickening in the vein graft had progressed remarkably by 4 weeks and that it had reached a peak at this time.[24,30] Our present results confirm the time course of intimal thickening in the rabbit model[22] and are also consistent with the previous report on VSMC proliferation as evaluated by PCNA staining.[26,27] It is important to note that in this study, the long-term inhibition of Rho kinase with fasudil suppressed the development of intimal thickening as well as VSMC proliferation, thus indicating an involvement of Rho kinase–mediated VSMC proliferation. Indeed, it has been reported that long-term inhibition of Rho kinase by either a Rho kinase inhibitor[33,34] or in vivo gene transfer of dominant-negative Rho kinase[14] suppresses intimal thickening after balloon injury.

VSMC migration from the media into the intima also plays an important role in intimal thickening in arteriosclerosis.[4,5] MLC phosphorylation, focal adhesion complex, and actin stress fiber formation, all of which are regulated by Rho kinase, may be involved in VSMC migration.[10-13] Although we did not specifically address this point in this study, it is possible that VSMC migration was also inhibited, because Rho kinase inhibitors suppress VSMC migration in vivo and in vitro.[35-37]

Another possible mechanism for the inhibition of intimal thickening by fasudil is the enhancement of VSMC apoptosis. It has been demonstrated in the experimental animal model that the regression of intimal thickening is associated with enhanced VSMC apoptosis.[38,39] We have recently demonstrated in a porcine model of in-stent restenosis that blockade of Rho kinase with fasudil enhances VSMC apoptosis, thus resulting in the suppression of the restenosis.[20] Thus, it is highly possible that apoptosis also plays a role in the suppression of intimal thickening of vein grafts.

We previously showed that long-term inhibition of Rho kinase by hydroxyfasudil or gene transfer of dominant-negative Rho kinase induced a marked regression of the constructive remodeling of porcine coronary artery in the experimental model.[11,15,17,40] However, in the vein graft, the external elastic lamina was unclear, and thus the external elastic lamina length could not be precisely measured. We examined the inside diameter of the internal elastic lamina for the surrogate measure, but there was no significant difference between the groups. This suggested that constructive remodeling may not play an important role in an autologous vein graft model.

In this study with an autologous vein graft model, macrophage infiltration was not substantially involved in the pathogenesis of intimal thickening. This is a finding similar to our previous study.[31] This suggested that macrophages may not play an important role to accelerate neointimal formation in normolipidemia.

In summary, we were able to demonstrate that the long-term inhibition of Rho kinase suppresses intimal thickening of autologous vein grafts in rabbits, for which inhibition of VSMC proliferation and enhancement of VSMC apoptosis may be involved. Thus, Rho kinase inhibitors may be useful for the prevention of vein graft disease.

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AUTHOR CONTRIBUTIONS

Conception and design: KK, HS
Analysis and interpretation: TF, YM, TU
Data collection: TF, YM, KH
Writing the article: TF, HS
Critical revision of the article: TF, KK, HS, KH
Final approval of the article: TF, KK, HS, YM, TU, KH, YM
Statistical analysis: TF, YM, TU
Obtained funding: TF, KK, YM
Overall responsibility: KK

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