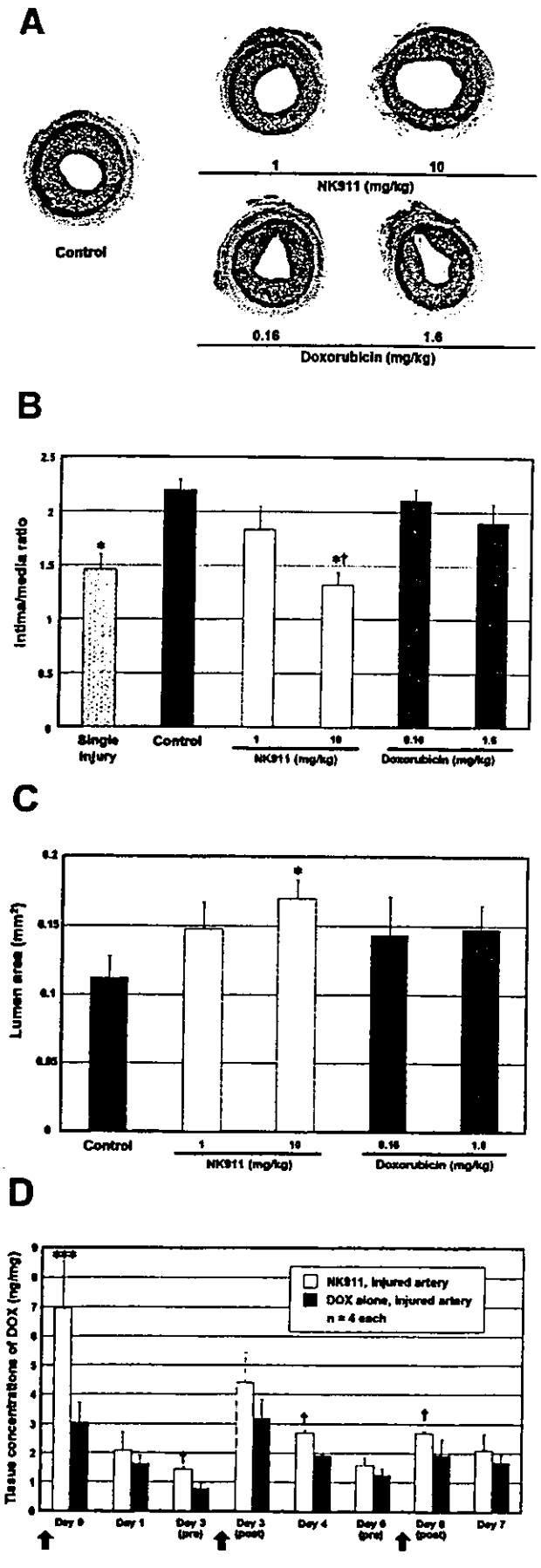


concentrations were 4-fold higher in the NK911 group than in the doxorubicin-alone group (Figure 2D).

Inhibitory Effect of NK911 in a Double-Injury Model

We further examined whether NK911 also inhibits vascular lesion formation in a double-injury model. The carotid artery lesion was induced by a balloon injury 2 weeks before a second balloon injury. At 4 weeks after the second injury, NK911 again inhibited the neointimal formation compared with the control group (Figures 3A and 3B) and maintained the lumen area at its maximum dose (Figure 3C). Again, neither NK911 nor doxorubicin affected the vascular remodeling (data not shown). In this double-injury model, NK911 again delivered doxorubicin to the balloon-injured artery more effectively than intravenous administration of the drug alone (Figure 3D). Thus, the efficacy of NK911 for the prevention of restenosis after balloon injury has been confirmed in those two models.

Mechanisms for the Inhibitory Effects of NK911

We then attempted to elucidate the mechanisms for the inhibitory effect of NK911 on neointimal formation after balloon injury. For this purpose, we performed immunostainings for PCNA, TUNEL, and ED-1, and RNA protection assay (RPA) for the expressions of cytokines and apoptosis-related molecules. The number of total cells in the injured carotid arteries was significantly decreased in the NK911 group as compared with the control or doxorubicin alone group in all 3 layers (Figure 4A). Furthermore, the inhibitory effect of NK911 was due in part to the inhibition of VSMC proliferation (Figure 4B) rather than enhancement of VSMC apoptosis (Figure 4C) or inhibition of macrophage recruitment (Figure 4D). The RPA analysis further demonstrated that NK911 significantly suppressed the expressions of several cytokines (eg, IL-1α, IL-6, IL-10, and TNF-β) (Figure 5A) but did not affect the expressions of apoptosis-related molecules (Figure 5B). These results are consistent with the findings with the PCNA and TUNEL stainings.

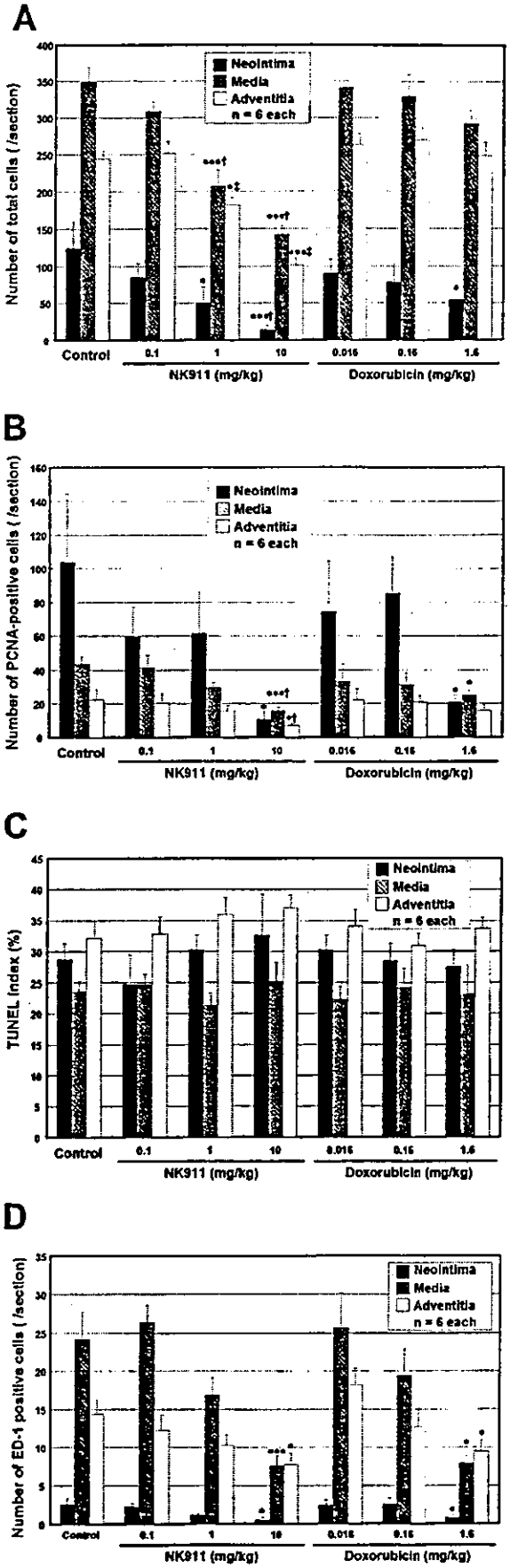
Side Effects of NK911

NK911 was well tolerated, and no side effects of NK911 were noted in terms of the time course of body weight (Table 1), hemodynamic variables, or liver/renal functions at 4 weeks after the NK911 treatment (Table 2). No abnormality was also found in hematology at 1 (data not shown) or 4 weeks (Table 2) after the treatment.

Discussion

The present study demonstrates that NK911, a nanoparticle carrier conjugated with doxorubicin, may be an effective and

Figure 3. Inhibitory effects of NK911 in a double-injury model. A, Photomicrographs (H&E staining) of the balloon-injured rat carotid artery. Top, NK911 group. Bottom, Doxorubicin alone group. B and C, Intima/media ratio (B) and lumen area (C) of the balloon-injured rat carotid artery (n=6 each). D, Tissue doxorubicin (DOX) concentrations in the balloon-injured rat carotid arteries. Arrows indicate the injection timing of NK911 or doxorubicin. Results are expressed as mean±SEM. *$P<0.05$ vs control group; ***$P<0.001$ vs all other groups; †$P<0.05$ vs doxorubicin alone group at a corresponding concentration.



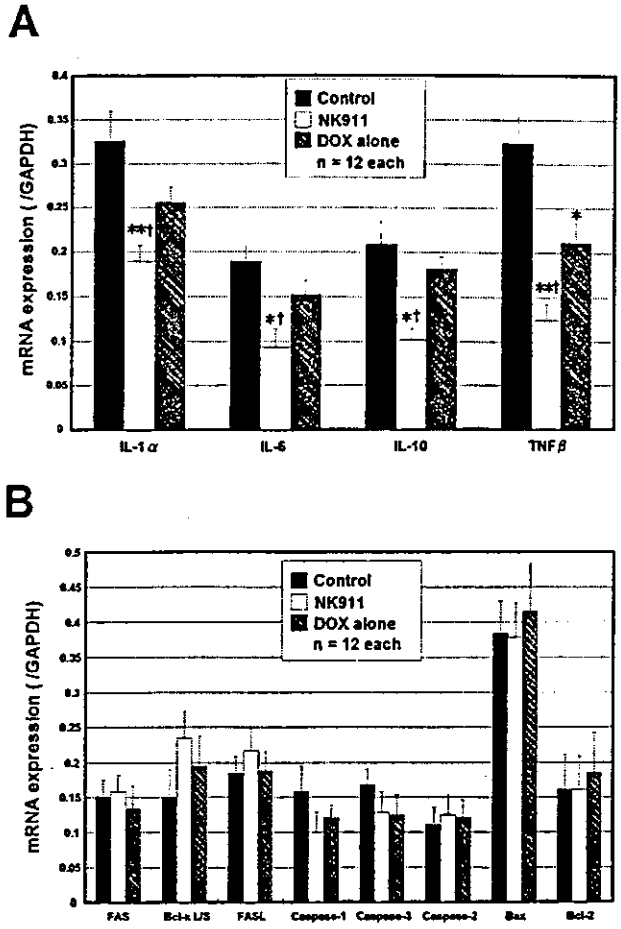


Figure 5. RNA protection assay for cytokines and apoptosis-related molecules. RNA protection assay for cytokines (A) and apoptosis-related molecules (B). Results are expressed as mean±SEM. *$P<0.05$, **$P<0.01$ vs control group; †$P<0.05$ vs doxorubicin (DOX) alone group.

safe treatment for the prevention of restenosis after balloon injury.

EPR Effect

EPR effect was first recognized in tumor tissues.[14] Most solid tumors have elevated levels of several factors that enhance vascular permeability, such as bradykinin, nitric oxide, peroxynitrite, prostaglandin, VEGF, and matrix metalloproteinases.[23] Furthermore, high vascular density and impaired lymphatic drainage enhance the accumulation of delivered agents.[23] Enhanced vascular permeability is also observed in granuloma and inflammatory and infected tissues.[24–27] After balloon injury, endothelial cells are removed, and consequently, local inflammation occurs at the balloon-injured site. In the present study with balloon injury in the rat carotid

Figure 4. Mechanisms for the inhibitory effects of NK911 on neointimal formation after balloon injury. A, Total cell count per section at 1 week after balloon injury in the rat carotid arteries. B through D, Number of cells (per section) positive for PCNA (B), TUNEL (C), or ED-1 (D) immunostaining at 1 week after the injury. Results are mean±SEM. *$P<0.05$, ***$P<0.001$ vs control group; †$P<0.05$, ‡$P<0.001$ vs doxorubicin alone group at a corresponding concentration.

TABLE 1. Time Course of Body Weight (kg)

	Week 0	Week 1	Week 2	Week 3	Week 4
Control	296±11	292±8	311±11	319±10	324±11*
Doxorubicin, mg/kg					
0.016	294±4	297±5	303±5	314±5	325±4*
0.16	295±5	302±5	312±4	320±4	328±5*
1.6	285±5	293±6	300±7	311±6	323±4*
NK911, mg/kg					
0.1	295±11	300±9	316±11	324±10	325±12*
1	282±5	286±2	301±2	315±6	311±5*
10	295±9	298±10	307±8	320±10	321±8*

Results are expressed as mean±SEM (n=6 each). Control indicates control group without any treatment; doxorubicin, doxorubicin alone group; and NK911, NK911 group. *$P<0.05$ vs week 0.

artery, we also confirmed the sustained vascular hyperpermeability, which should facilitate the efficient delivery of doxorubicin by NK911 to the balloon-injured artery in vivo.

Selective Delivery of Doxorubicin by NK911 In Vivo

NK911 is observed to accumulate in the vascular lesion where permeability is increased. The NK911 accumulation may be mediated by two mechanisms; first, the size of the micelle may be adequate for enhanced accumulation. Indeed, it was demonstrated that the size of the micelle is critical for accumulation of the nanoparticle and that oversized micelles may result in reduced tissue accumulation.[28] Second, the surface charge of the micelle may be important. The surface of NK911 is negatively charged, whereas the luminal surface of the injured blood vessel is positively charged. Therefore, the NK911 accumulation is accelerated in the balloon-injured artery with a lower entrapment rate by the liver or the spleen when compared with the micelle with a neutral charge.[29]

In this study, NK911 significantly suppressed the vascular lesion formation after balloon injury more effectively than the drug alone (0.16 mg/kg content of doxorubicin) at lower concentrations required for the treatment of cancers (0.2 to 0.6 mg/kg, administered intravenously, 3 to 4 times a week, repeated 2 to 3 times). Furthermore, measurement of tissue concentrations of doxorubicin confirmed the effectiveness of NK911 to selectively deliver the agent to the balloon-injured artery in vivo. Because the most significant difference in the tissue doxorubicin concentrations was noted immediately (3 hours) after balloon injury, it is conceivable that a single intravenous administration of NK911 might be enough to suppress the subsequent vascular lesion formation, although this point remains to be examined in a future study.

Mechanisms for the Inhibitory Effect of NK911 on Neointimal Formation After Balloon Injury In Vivo

It was recently suggested that one of the antiproliferative effects of doxorubicin is mediated by enhanced apoptosis through several apoptotic pathway.[30] However, it was also reported that doxorubicin damages the cell membrane but does not induce cell apoptosis, unlike other anthracycline family members.[31] In this study, NK911 inhibited vascular proliferation but did not enhance apoptosis. Indeed, NK911 suppressed neointimal formation and the expression of several cytokines that promote VSMC proliferation. Adriamycin downregulates the expression of the cyclin D1, the major regulator of cell cycle into the proliferative stage in several tumor cell lines.[32] It was also reported that doxorubicin suppressed the expression of oncogene c-myc and c-jun in rat

TABLE 2. Hemodynamic Variables, Liver/Renal Functions, and Blood Analysis (4 Weeks After Balloon Injury)

		Doxorubicin, mg/kg			NK911, mg/kg		
	Control	0.016	0.16	1.6	0.1	1	10
BP, mm Hg	127±4	126±3	128±4	128±2	127±3	122±6	129±3
HR, bpm	318±16	314±4	315±5	312±4	312±8	317±9	307±7
Liver functions							
T. Bil, mg/dL	0.13±0.02	0.12±0.02	0.12±0.02	0.17±0.03	0.14±0.05	0.16±0.05	0.15±0.06
AST, IU/L	141±10	124±26	118±19	136±23	132±9	122±10	131±15
ALT, IU/L	42±5	34±3	34±2	36±3	37±2	38±3	41±5
Renal functions							
BUN, mg/dL	20±1	20±2	17±2	18±2	21±2	20±2	17±1
Cr, mg/dL	0.26±0.01	0.26±0.01	0.24±0.02	0.24±0.01	0.28±0.02	0.27±0.02	0.29±0.04
Blood analysis							
WBC, per μL	2772±280	2283±317	2267±211	2583±381	3117±457	2433±158	2350±145
RBC, $\times 10^4/\mu L$	834±29	801±24	852±22	801±17	799±30	847±26	791±12
Hb, g/dL	14.8±0.4	14.1±0.5	14.1±0.3	14.0±0.3	14.1±0.5	14.9±0.4	14.3±0.4
Ht, %	43.1±1.6	40.1±1.6	40.9±1.8	40.3±1.4	42.7±1.9	44.6±1.4	42.8±1.0
Plts, $\times 10^4/\mu L$	43.4±4.3	51.2±5.9	52.9±3.6	45.6±3.3	48.6±5.4	52.5±4.8	47.3±6.1

Results are expressed as mean±SEM (n=6 each). Control indicates control group without any treatment; doxorubicin, doxorubicin alone group; NK911, NK911 group; T. Bil, total bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; Cr, creatinine; WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin; Ht, hematocrit; and Plts, platelets.

glioblastoma cell line.[33] Furthermore, doxorubicin directly inhibits the release of cytokine (eg, IL-6, IFN-γ) from stimulated peripheral mononuclear cell at its nontoxic concentration in vitro.[34,35] These mechanisms may contribute to the inhibition of VSMC proliferation by NK911 after balloon injury. In contrast, NK911 did not affect vascular remodeling. Thus, NK911 may be more useful for the prevention of vascular lesion formation after coronary stenting rather than that after balloon angioplasty alone, because the contribution of neointimal formation to restenotic vascular lesion formation is greater after stenting than after balloon injury alone.[36]

Safety of NK911

In this study, NK911 caused no side effects while it significantly suppressed the restenotic changes of the carotid artery after balloon injury. Indeed, it was previously confirmed that NK911 causes no damage of major organs when examined histologically.[37] Furthermore, it was previously shown that the cardiotoxicity of doxorubicin is markedly reduced when selectively delivered in NK911.[38] It has been recently reported that NK911 exerts anticancer effects at a dose of up to 24 mg/kg without any major side effects.[13] In this study, we were also able to rule out the involvement of the potential cytotoxic effect of doxorubicin in the biological effect of NK911.

Limitations of the Study

Several limitations of the present study should be mentioned. First, the effect of bare micelle alone was not examined in this study. NK911 consists of PEG-conjugated doxorubicin in the micelle that is pharmacologically ineffective and free doxorubicin incorporated inside the micelle that is pharmacologically effective.[39,40] Both components of doxorubicin are required for the stability of NK911.[39,40] Thus, the "bare micelle" alone may not be a suitable control for NK911, and instead, we examined the effect of doxorubicin alone as a control in this study. Second, the double-injury model in this study may not represent atherosclerotic blood vessels in humans. Thus, the inhibitory effects of NK911 should be tested in atherosclerotic animal models in primates before its application to humans. Third, the frequencies of TUNEL- or PCNA-positive cells were relatively high in this study although the results are consistent with those of the previous studies.[19,20] Thus, we consider that the frequencies may not represent the actual rate of apoptosis or proliferation but may rather reflect the relative extent of those processes. Fourth, doxorubicin has a potential cytotoxicity that may affect DNA function with resultant carcinogenicity. Although intravenous administration of the agent just 3 times appears to be enough to suppress the vascular lesion formation, it may be more appropriate to deliver antiproliferative agents with less cytotoxicity by using nanoparticles. Fifth, NK911 is a first-generation nanoparticle that utilizes only the EPR effects of balloon-injured artery. More sophisticated nanoparticle systems need to be developed. Indeed, with recent advances in nanotechnology, several nanoparticles that can specifically recognize surface antigens or differences in tissue composition or temperature have already been developed.[41–43]

In summary, the present study demonstrates that nanoparticle technology targeting balloon-injured arteries with increased permeability is a promising and safe approach for the prevention of restenosis after the procedure.

Acknowledgments

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Extracorporeal Cardiac Shock Wave Therapy Markedly Ameliorates Ischemia-Induced Myocardial Dysfunction in Pigs in Vivo

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Background—Prognosis of ischemic cardiomyopathy still remains poor because of the lack of effective treatments. To develop a noninvasive therapy for the disorder, we examined the in vitro and vivo effects of extracorporeal shock wave (SW) that could enhance angiogenesis.

Methods and Results—SW treatment applied to cultured human umbilical vein endothelial cells significantly upregulated mRNA expression of vascular endothelial growth factor and its receptor Flt-1 in vitro. A porcine model of chronic myocardial ischemia was made by placing an ameroid constrictor at the proximal segment of the left circumflex coronary artery, which gradually induced a total occlusion of the artery with sustained myocardial dysfunction but without myocardial infarction in 4 weeks. Thereafter, extracorporeal SW therapy to the ischemic myocardial region (200 shots/spot for 9 spots at 0.09 mJ/mm^2) was performed (n=8), which induced a complete recovery of left ventricular ejection fraction (51±2% to 62±2%), wall thickening fraction (13±3% to 30±3%), and regional myocardial blood flow (1.0±0.2 to 1.4±0.3 $mL \cdot min^{-1} \cdot g^{-1}$) of the ischemic region in 4 weeks (all $P<0.01$). By contrast, animals that did not receive the therapy (n=8) had sustained myocardial dysfunction (left ventricular ejection fraction, 48±3% to 48±1%; wall thickening fraction, 13±2% to 9±2%) and regional myocardial blood flow (1.0±0.3 to 0.6±0.1 $mL \cdot min^{-1} \cdot g^{-1}$). Neither arrhythmias nor other complications were observed during or after the treatment. SW treatment of the ischemic myocardium significantly upregulated vascular endothelial growth factor expression in vivo.

Conclusions—These results suggest that extracorporeal cardiac SW therapy is an effective and noninvasive therapeutic strategy for ischemic heart disease. (***Circulation.* 2004;110:3055-3061.**)

Key Words: angiogenesis ■ contractility ■ hibernation ■ ischemia ■ regional blood flow

Prognosis of ischemic cardiomyopathy without an indication for coronary intervention or coronary artery bypass grafting still remains poor because medication is the only therapy to treat the disorder.[1] Thus, it is imperative that an effective and noninvasive therapy for ischemic cardiomyopathy be developed. Although no medication or procedure used clinically has shown efficacy in replacing myocardial scar with functioning contractile tissue, it could be possible to improve the contractility of the hibernating myocardium by inducing angiogenesis.

It recently has been suggested that shock wave (SW) could enhance angiogenesis in vitro.[2] SW is a longitudinal acoustic wave, traveling with the speed in water of ultrasound through body tissue. It is a single pressure pulse with a short needle-like positive spike <1 μs in duration and up to 100 MPa in amplitude, followed by a tensile part of several microseconds with lower amplitude.[3] SW is known to exert the "cavitation effect" (a micrometer-sized violent collapse of bubbles inside and outside the cells)[3] and recently has been demonstrated to induce localized stress on cell membranes that resembles shear stress.[4] If SW-induced angiogenesis could be reproduced in vivo, it would provide a unique opportunity to develop a new angiogenic therapy that would not require invasive procedures such as open-chest surgery or catheter intervention. Therefore, the present study was designed to examine the possible beneficial effects of SW on ischemia-induced myocardial dysfunction in a porcine model of chronic myocardial ischemia in vivo.

Methods

This study was reviewed by the Committee on Ethics in Animal Experiments of Kyushu University and was carried out under the

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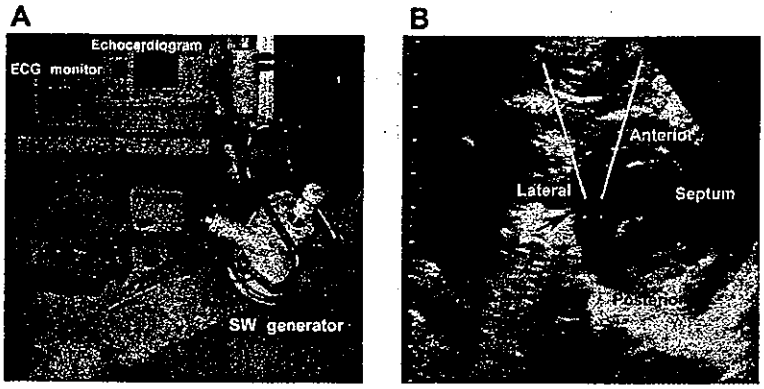

Figure 1. Extracorporeal cardiac SW therapy in action in a pig chronically instrumented with an ameroid constrictor. A, The machine is equipped with a SW generator and in-line echocardiography. The SW generator is attached to the chest wall when used. B, The SW pulse is easily focused on the ischemic myocardium under the guidance of echocardiography (black arrow).

Guidelines for Animal Experiments of Kyushu University and the Law (No. 105) and Notification (No. 6) of the Japanese Government.

Effect of SW on Human Umbilical Vein Endothelial Cells in Vitro

We purchased single-donor human umbilical vein endothelial cells (HUVECs) (Clonetics, Walkersville, Md) and cultured them in a complete endothelial medium (EBM-2 BulletKit, Clonetics). HUVECs were subcultured and used at passages 3 to 5 and were maintained in EBM-2. Twenty-four hours before the SW treatment, HUVECs (1×10^5) were resuspended in a 2-mL tube with EBM (Clonetics). We treated the HUVECs with 500 shots of SW at 4 different energy levels (0 [control], 0.02, 0.09, 0.18, and 0.35 mJ/mm^2) and stored them for 24 hours in the same medium before RNA extraction.

Ribonuclease Protection Assay

We analyzed equal amounts of mRNA by ribonuclease protection assay by means of the RiboQuant multiprobe template (PharMingen). Briefly, we hybridized RNA overnight with a ^{32}P-labeled RNA probe, which previously had been synthesized from the template set. We digested single-stranded RNA and free probe by ribonuclease A and T1. We then analyzed protected RNA on a 5% denaturing polyacrylamide gel. We analyzed several angiogenic factors, including vascular endothelial growth factor (VEGF) and its receptor, *fms*-like tyrosine kinase (Flt)-1, and angiopoietin and its receptor, tie-1, either by means of an NIH image or by means of autoradiography and subsequent quantification by densitometry (Alpha Innotech). For quantification, we normalized the signals for each sample of the blot with the corresponding signals of the housekeeping genes GAPDH and L32.

Porcine Model of Chronic Myocardial Ischemia

A total of 28 domestic pigs (25 to 30 kg in body weight) were used in this study. We anesthetized the animals with ketamine (15 mg/kg IM) and maintained anesthesia with an inhalation of 1.5% isoflurane for implantation of an ameroid constrictor, SW treatment, and euthanization. We opened the chest, suspended the pericardium and the left atrial appendage, revealed the left circumflex coronary artery (LCx), and put an ameroid constrictor around the proximal LCx to gradually induce a total occlusion of the artery in 4 weeks without causing myocardial infarction.[5,6] We also confirmed histologically that no myocardial necrosis had developed in the present porcine model (data not shown). This model is widely used to examine the effect of an angiogenic therapy in the ischemic hibernating myocardium.[5,6]

Extracorporeal Cardiac SW Therapy to Chronic Ischemic Myocardium

On the basis of the in vitro experiment, we applied a low energy of SW (0.09 mJ/mm2, ≈10% of the energy for the lithotripsy treatment) to 9 spots in the ischemic region (200 shots/spot) with the guidance of an echocardiogram equipped within a specially designed SW generator (Storz Medical AG) (Figure 1A). We were able to focus SW in any part of the heart under the guidance of echocardiography (Figure 1B). We applied SW to the ischemic myocardium in an R-wave–triggered manner to avoid ventricular arrhythmias. We performed the SW treatment (n=8) at 4 weeks after the implantation of an ameroid constrictor 3 times within 1 week, whereas animals in the control group (n=8) received the same anesthesia procedures 3 times a week but without the SW treatment. Because the SW treatment only requires the gentle compression of the generator to the chest wall, it is unlikely that this handling itself enhances angiogenesis in the ischemic myocardium.

Coronary Angiography and Left Ventriculography

After systemic heparinization (10 000 U/body), we performed coronary angiography and left ventriculography in a left oblique view with the use of a cineangiography system (Toshiba Medical). We semiquantitatively evaluated the extent of collateral flow to the LCx by the graded Rentrop score (0, no visible collateral vessels; 1, faint filling of side branches of the main epicardial vessel without filling the main vessel; 2, partial filling of the main epicardial vessel; 3, complete filling of the main vessel).[7] We also counted the number of visible coronary arteries in the LCx region. To compare the extent of collateral development at a given time, we selected the frame in which the whole left anterior descending coronary artery was first visualized.

Echocardiographic Evaluation

We performed epicardial echocardiographic studies at ameroid implantation (baseline) and at 4 and 8 weeks after the implantation of the constrictor (Sonos 5500, Agilent Technology). We calculated wall thickening fraction (WTF) by using the following formula: WTF = 100 × (end-systolic wall thickness − end-diastolic wall thickness) /end-diastolic wall thickness. We measured WTF when pigs were sedated, with and without dobutamine loading (15 $\mu g \cdot kg^{-1} \cdot min^{-1}$). Dobutamine was infused continuously from the ear vein, and WTF was measured after the hemodynamic condition was stabilized (in ≈5 minutes).

Measurement of Regional Myocardial Blood Flow

We evaluated regional myocardial blood flow (RMBF) with colored microspheres (Dye-Trak, Triton Technology) at ameroid implantation (baseline) and at 4 and 8 weeks after implantation.[8] We injected microspheres through the left atrium and aspirated a reference arterial blood sample from the descending aorta at a constant rate of 20 mL/min for 60 seconds using a withdrawal pump. We extracted microspheres from the left ventricular (LV) wall and blood samples by potassium hydroxide digestion, extracted the dyes from the spheres with dimethylformamide (200 μL), and determined their concentrations by spectrophotometry.[8] We calculated myocardial blood flow ($mL \cdot min^{-1} \cdot g^{-1}$) of the endocardial and epicardial lateral LV wall (the LCx region).

Analysis of Cardiac Enzymes

We measured serum concentrations of cardiac troponin T and creatinine kinase (CK)–MB by using chemiluminescence immuno-

assay before the SW treatment and at 4, 5 (2 hours after the SW treatment), and 8 weeks after ameroid implantation.

Factor VIII Staining

We treated paraffin-embedded sections with a rabbit anti–factor VIII antibody (N1505, Dako, Copenhagen, Denmark). We counted the number of factor VIII–positive cells in the endocardial and epicardial wall in 10 fields of the LCx region in each heart at 400× magnification.

Real-Time Polymerase Chain Reaction

To examine the effect of SW treatment on the ischemic myocardium in vivo, the animals with an ameroid constrictor were euthanized 1 week after the SW treatment. Total RNA was isolated from rapidly frozen ischemic LV wall (LCx region) after 3 SW treatments and was reverse transcribed. Quantification of VEGF and its receptor Flt-1 was performed by amplification of cDNA with an ABI Prism 7000 real-time thermocycler.

Western Blot Analysis for VEGF

We performed Western blot analysis for VEGF. Western blot analysis for VEGF was performed with and without 3 SW treatments. Three sections from the ischemic LV wall (LCx region) were measured. The regions containing VEGF proteins were visualized by electrochemiluminescence Western blotting luminal reagent (Santa Cruz Biotechnology). The extent of the VEGF was normalized by that of β-actin.

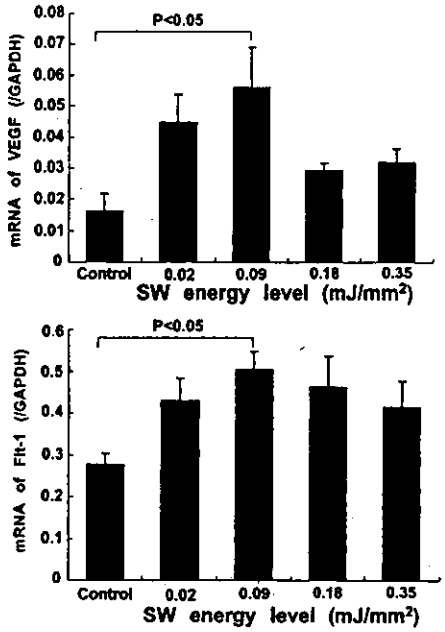

Figure 2. SW treatment upregulated mRNA expression of VEGF (A) and Flt-1 (B) in HUVECs in vitro with a maximum effect noted at 0.09 mJ/mm². Results are expressed as mean±SEM (n=10 each).

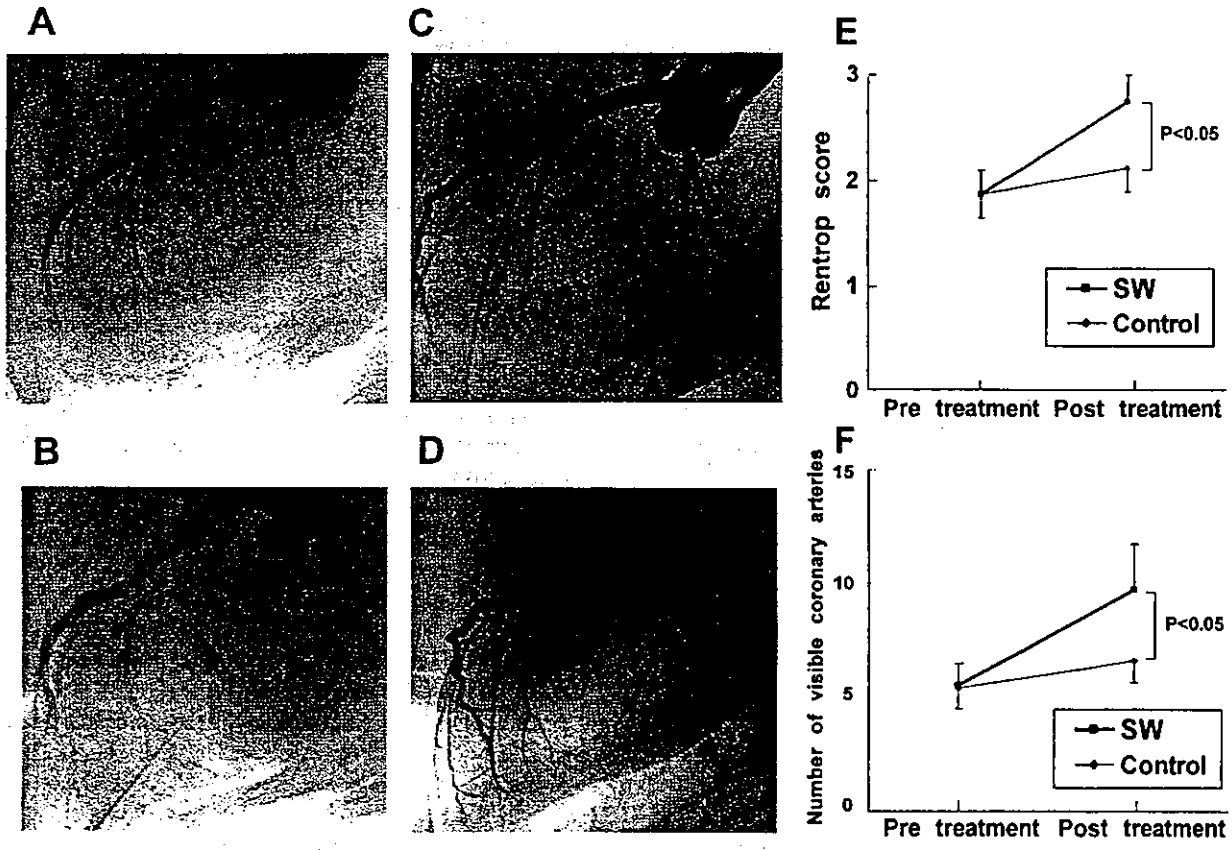

Figure 3. Extracorporeal cardiac SW therapy enhances coronary angiogenesis in vivo. A and C, Four weeks after the implantation of an ameroid constrictor, LCx was totally occluded and was perfused via collateral vessels with severe delay in both the control group (A) and the SW group (before SW therapy) (C). B and D, Four weeks after the first coronary angiography, no significant change in coronary vessels was noted in the control group (B), whereas a marked development of visible coronary vessels was noted in the SW group (D). E and F, Four weeks after the first coronary angiography, no significant increase in the Rentrop score (E) or visible coronary arteries from LCx (F) was noted in the control group, whereas increased Rentrop score and a marked development of visible coronary vessels were noted in the SW group. Results are expressed as mean±SEM (n=8 each).

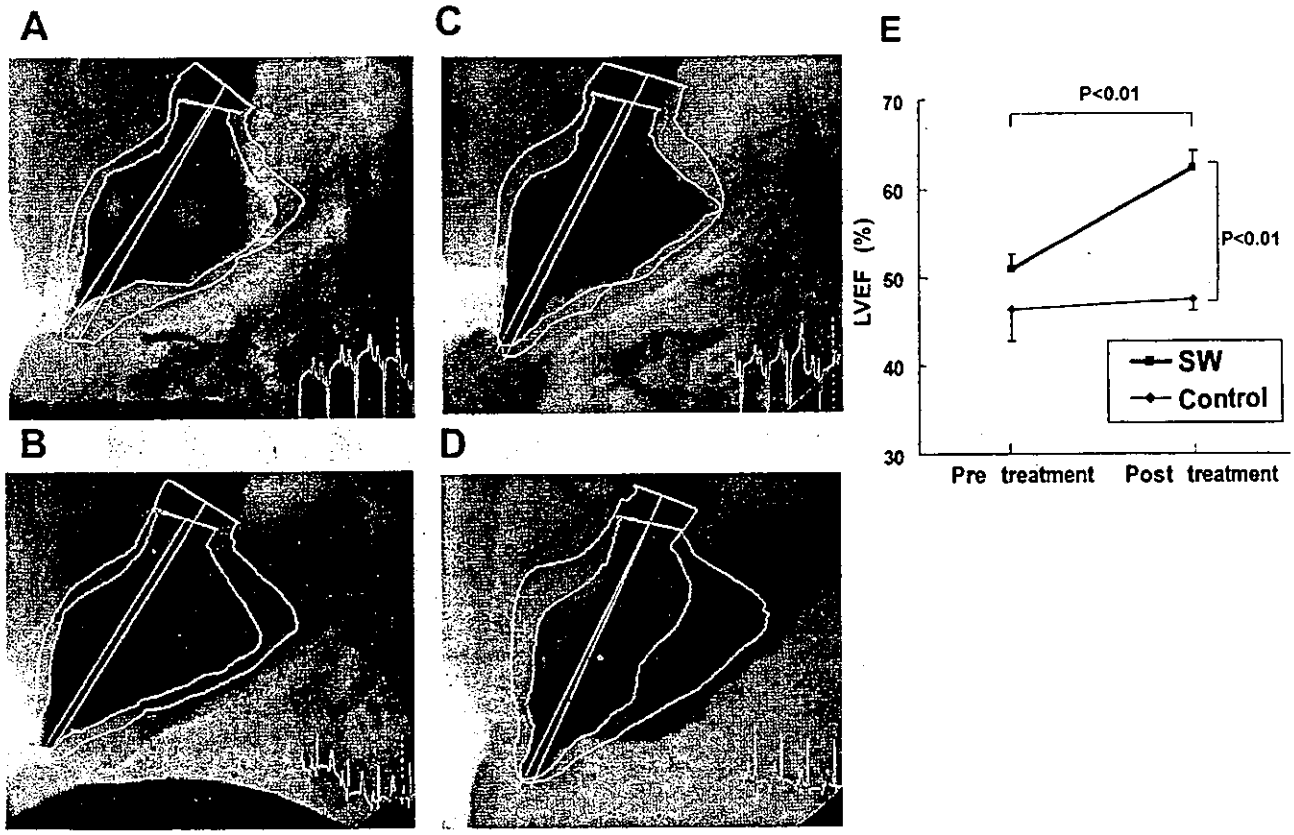

Figure 4. Extracorporeal cardiac SW therapy improves ischemia-induced myocardial dysfunction in vivo. A and C, Four weeks after the implantation of an ameroid constrictor, LV wall motion of the LCx (posterolateral) region was reduced in both the control (A) and the SW group (before the SW therapy) (C). B and D, Four weeks after the first left ventriculography, no significant change in LV wall motion was noted in the control group (B), whereas marked recovery was noted in the SW group (D). E, The SW therapy normalized left ventricular ejection fraction in the SW group but not in the control group. Results are expressed as mean±SEM (n=8 each).

Statistical Analysis

Results are expressed as mean±SEM. We determined statistical significance by analysis of variance for multiple comparisons. A value of $P<0.05$ was considered to be statistically significant.

Results

Effect of SW on mRNA Expression of VEGF and Flt-1 in HUVECs

SW treatment significantly upregulated mRNA expression of VEGF and its receptor Flt-1 in HUVECs, with a maximum effect noted at 0.09 mJ/mm^2 (Figure 2).

Effects of Extracorporeal Cardiac SW Therapy on Angiogenesis and Ischemia-Induced Myocardial Dysfunction

Four weeks after ameroid implantation, coronary angiography demonstrated a total occlusion of the LCx, which was perfused via collateral vessels with severe delay in both the control (Figure 3A) and the SW groups (Figure 3C). At 8 weeks after ameroid implantation (4 weeks after SW therapy), the SW group (Figure 3D), but not the control group (Figure 3B), had a marked development of coronary collateral vessels in the ischemic LCx region, an increased Rentrop score (Figure 3E), and an increased number of visible coronary arteries in the region (Figure 3F). Similarly, at 4 weeks, left ventriculography demonstrated an impaired left ventricular ejection fraction in both groups (Figure 4A, 4C, and 4E), whereas at 8 weeks, left ventricular ejection fraction was normalized in the SW group but remained impaired in the control group (Figure 4B, 4D, and 4E).

Effects of Extracorporeal Cardiac SW Therapy on Regional Myocardial Function and Myocardial Blood Flow

We serially measured WTF of the LCx region (lateral wall of the LV) by epicardiac echocardiography. At 4 weeks, we observed a significant reduction in WTF (%) in both groups (13±2 in the control group and 13±3 in the SW group; Figure 5A). At 8 weeks, however, the SW treatment markedly improved WTF in the SW group (30±3) but not in the control group (9±2) under control conditions (Figure 5A). Under dobutamine-loading conditions, which mimicked exercise conditions, WTF was further reduced at 4 weeks after the ameroid implantation in both groups (16±3 in the control and 18±2 in the SW groups), however, at 8 weeks, WTF was again markedly ameliorated only in the SW group (31±2) but not in the control group (16±4) (Figure 5B).

At 4 weeks, RMBF in the endocardium and epicardium (mL · min^{-1} · g^{-1}) was equally decreased in both groups (1.0±0.3 and 0.9±0.2 in the control group and 1.0±0.2 and 0.9±0.2 in the SW group, respectively). The SW treatment again improved RMBF in the endocardium (0.6±0.1 in the

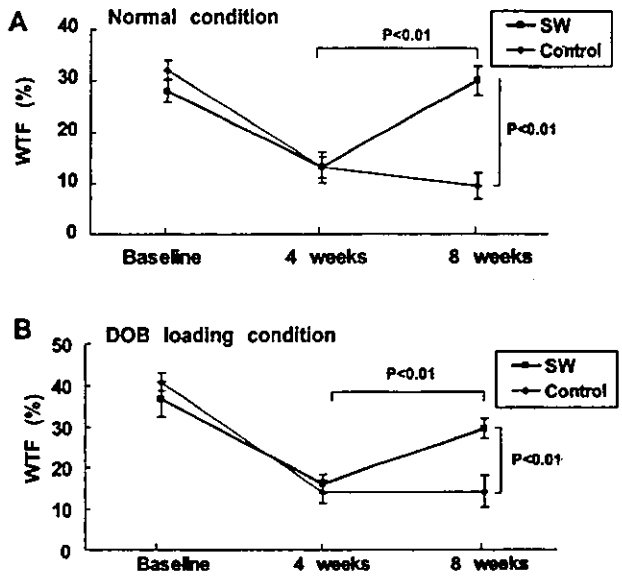

Figure 5. Extracorporeal cardiac SW therapy improves regional myocardial function in vivo. SW therapy induced a complete recovery of WTF of the ischemic lateral wall under control conditions (A) and under dobutamine (DOB) loading conditions (B). Results are expressed as mean±SEM (n=8 each).

control group and 1.4±0.3 in the SW group, $P<0.05$; Figure 6A) as well as in the epicardium (0.7±0.2 in the control group and 1.5±0.2 in the SW group, $P<0.05$; Figure 6B).

Effects of Extracorporeal Cardiac SW Therapy on Capillary Density and VEGF Expression in the Ischemic Myocardium

Factor VIII staining showed that the number of factor VIII–positive capillaries was increased in the SW group compared with the control group (Figure 7A and 7B). Quantitative analysis demonstrated that the number of capillaries was significantly higher in the SW group in both the endocardium (840±26 in the control group and 1280±45 in the SW group, $P<0.05$; Figure 7C) and the epicardium (820±30 in the control group and 1200±22 in the SW group, $P<0.05$; Figure 7D). RT-PCR analysis and Western blotting demonstrated a significant upregulation of VEGF mRNA expression (8.0±6 in the control group and 32±8 in the SW group, $P<0.05$; Figure 8A) and protein expression (2.23-fold increase in the SW groups, $P<0.05$; Figure 8B) after the SW treatment to the ischemic myocardium in vivo.

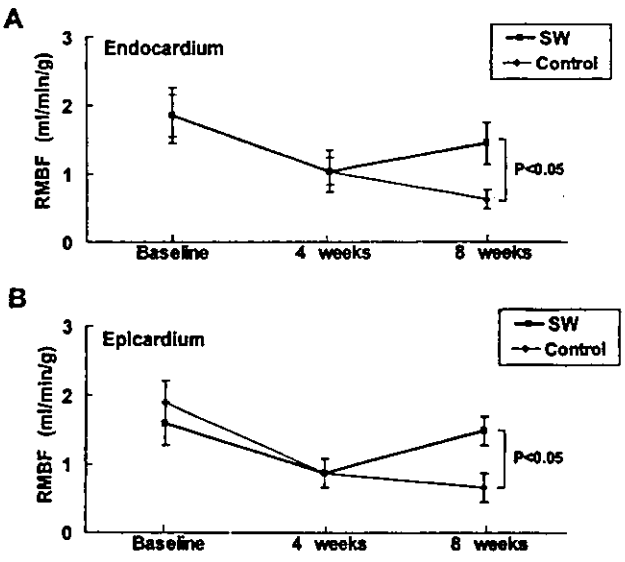

Figure 6. Extracorporeal cardiac SW therapy improves RMBF in vivo. SW therapy significantly increased RMBF, assessed by colored microspheres in both the endocardium (A) and the epicardium (B). Results are expressed as mean±SEM (n=8 each).

Side Effects of Extracorporeal Cardiac SW Therapy

All animals treated with the SW therapy were alive and showed no arrhythmias as assessed by 24-hour Holter ECG during and after the treatment (n=3; data not shown). There also was no myocardial cell damage as assessed by serum concentrations of CK-MB (ng/mL); the values before the SW treatment and at 4, 5 (2 hours after the SW treatment), and 8 weeks after the ameroid implantation were 5.0±0.6, 6.2±0.5, 5.5±0.2, and 7.1±0.9 in the control group and 5.1±0.2, 7.7±0.6, 6.1±0.6, and 6.4±0.4 in the SW group, respectively (n=6 each). The serum concentrations of troponin T were not detected in most cases in both groups. No significant differences were noted in hemodynamic variables (eg, heart rate or blood pressure) between the 2 groups (data not shown).

Discussion

The novel finding of the present study is that the extracorporeal cardiac SW therapy enhances angiogenesis in the ischemic myocardium and normalizes myocardial function in a porcine model of chronic myocardial ischemia in vivo. To the best of our knowledge, this is the first report that demonstrates the potential usefulness of extracorporeal cardiac SW therapy as a noninvasive treatment of chronic myocardial ischemia.

Extracorporeal Cardiac SW Therapy as a Novel Strategy for Ischemic Cardiomyopathy

Because of the poor prognosis of ischemic cardiomyopathy,[1,9] it is crucial to develop an alternative therapy for ischemia-induced myocardial dysfunction. To accomplish effective angiogenesis, it is mandatory to upregulate potent angiogenesis ligands, such as VEGF, and their receptors.[9,10] Furthermore, in the clinical setting, the goal for the treatment of ischemic cardiomyopathy should include not only enhancement of angiogenesis but also recovery of ischemia-induced myocardial dysfunction. In the present study, we were able to demonstrate that SW treatment (1) normalized global and regional myocardial functions as well as RMBF of the chronic ischemic region without any adverse effects in vivo, (2) increased vascular density in the SW-treated region, and (3) enhanced mRNA expression of VEGF and its receptor Flt-1 in HUVECs in vitro and VEGF production in the ischemic myocardium in vivo. Thus, SW-induced upregulation of the endogenous angiogenic system may offer a novel and promising noninvasive strategy for the treatment of ischemic heart disease.

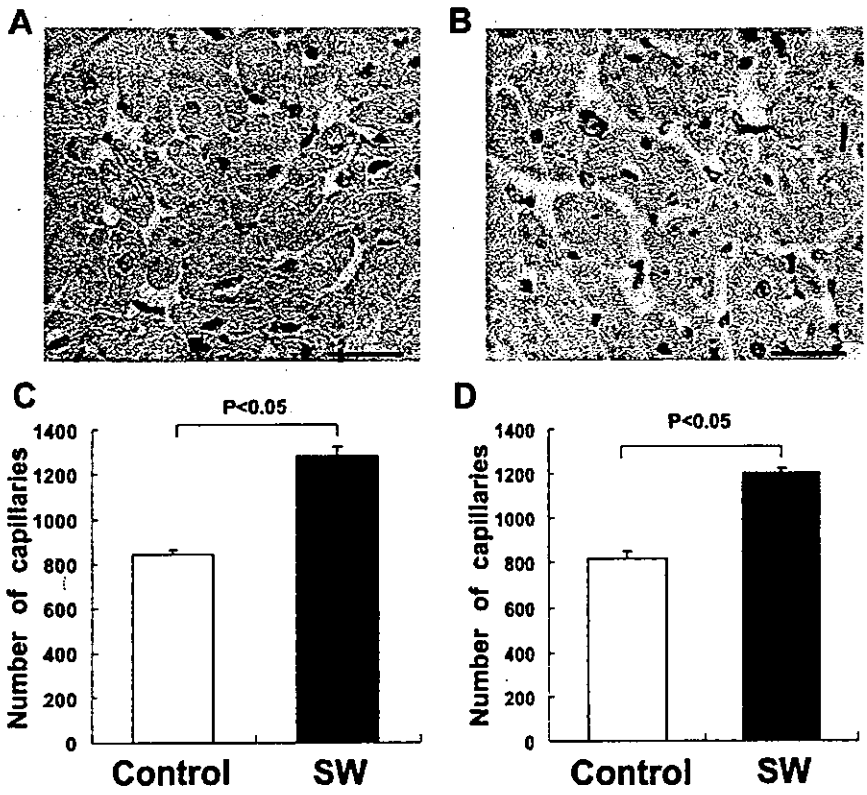

Figure 7. Extracorporeal cardiac SW therapy increases the density of factor VIII–positive capillaries in the ischemic myocardium. A and B, Factor VIII staining of the LCx region from the control (A) and the SW group (B). Scale bar represents 20 μm. C and D, Capillary density was significantly greater in the SW group (SW) than in the control group (Control) in both the endocardium (C) and the epicardium (D). Results are expressed as mean±SEM (n=6 each).

Advantages of Extracorporeal Cardiac SW Therapy

Recent attempts to enhance angiogenesis in the ischemic organs include gene therapy and bone marrow cell transplantation therapy. The main purpose of gene therapy is to induce overexpression of a selected angiogenic ligand (eg, VEGF) that leads to angiogenesis in the ischemic region. Although phase 1 trials of gene transfer of plasmid DNA encoding VEGF demonstrated safety and clinical benefit for the treatment of ischemic limb and heart,[11–13] gene therapy for ischemic cardiomyopathy is still at a preclinical stage. Bone marrow cell transplantation therapy, which depends on adult stem cell plasticity, also may be a useful strategy for angiogenesis because endothelial progenitor cells could be isolated from circulating mononuclear cells in humans and could be shown to be incorporated into neovascularization.[14] However, the need for invasive delivery of those cells to the ischemic myocardium may severely limit its usefulness in clinical situations.

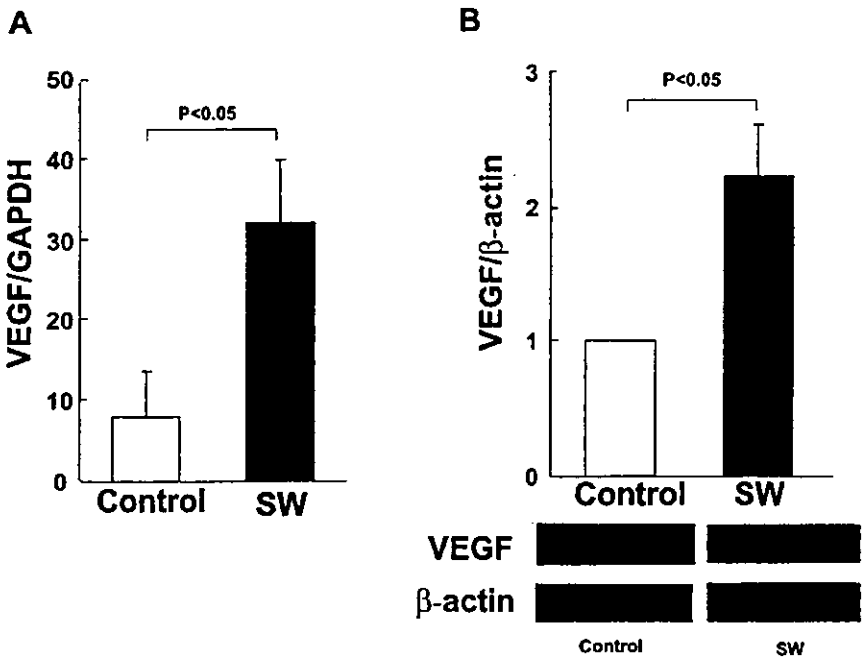

Figure 8. SW treatment upreglated mRNA (A) and protein (B) expression of VEGF in the ischemic myocardium (n=5 each).

A major advantage of the extracorporeal cardiac SW therapy over these 2 strategies is shown by the fact that it is quite noninvasive and safe, without any adverse effects. If necessary, we could repeatedly treat patients (even outpatients) with SW therapy because no surgery, anesthesia, or even catheter intervention is required for the treatment. This is an important factor in determining the clinical usefulness of angiogenic therapies in patients with ischemic cardiomyopathy. Thus, the extracorporeal cardiac SW therapy appears to be an applicable and noninvasive treatment for ischemic heart disease. Indeed, the SW treatment itself already has been clinically established as an effective and safe treatment for lithotripsy and chronic plantar fasciitis.[15,16] Our present results indicate that SW therapy, at ≈10% of the energy needed for lithotripsy treatment, is effective for in vivo angiogenesis in the ischemic heart.

Mechanisms for SW-Induced Angiogenesis

When a SW hits tissue, cavitation (a micrometer-sized violent collapse of bubbles) is induced by the first compression by the positive pressure part and the expansion with the tensile part of a SW.[3] Because the physical forces generated by cavitation are highly localized, SW could induce localized stress on cell membranes, as altered shear stress affects endothelial cells.[17] Recent reports have demonstrated the biochemical effects of SW, including hyperpolarization and Ras activation,[18] nonenzymatic nitric oxide synthesis,[19] and induction of stress fibers and intercellular gaps.[20] Although precise mechanisms for the SW-induced biochemical effects remain to be examined, these mechanisms may be involved in the underlying mechanisms for SW-induced angiogenesis. Indeed, Wang et al[21] reported that SW induces angiogenesis of the Achilles tendon–bone junction in dogs.

We were able to demonstrate that the SW treatment upregulated mRNA expression of VEGF and its receptor Flt in HUVECs in vitro and VEGF expression in the ischemic myocardium in vivo. Because the VEGF-Flt system is essential in initiating vasculogenesis and/or angiogenesis,[22] this effect of SW could explain, at least in part, the underlying mechanisms for SW-induced angiogenesis. It should be noted, however, that we showed only the upregulation of VEGF and Flt and that the effect of SW on signal transduction after receptor–ligand interaction still remains to be clarified. In addition, we need to fully elucidate the mechanisms for the SW-induced complete recovery of ischemia-induced myocardial dysfunction, although the increased myocardial blood flow caused by the SW treatment appears to play a primary role for the improved myocardial function. Further studies are required to determine the precise molecular mechanism for SW-induced angiogenesis and recovery of myocardial function.

In summary, we were able to demonstrate that noninvasive extracorporeal cardiac SW therapy effectively increases RMBF and normalizes ischemia-induced myocardial dysfunction without any adverse effects. Thus, extracorporeal cardiac SW therapy may be an effective, safe, and noninvasive therapy for ischemic cardiomyopathy.

Acknowledgments

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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 autoimmunity, 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-2^k, aged 9 to 11 weeks) were used as heart donors, and C3H/He (H-2^k) 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

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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 1000 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 μmol/L 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 (2.3×10^9 pfu/mL in 0.15 mL as a control) were transfected to allografts as previously described.[16] In a preliminary study, we confirmed the expression of the LacZ gene throughout the heart by X-gal staining 1 week after the transplantation. After 4 weeks, the grafts were harvested and the extent of CAV was analyzed histologically.

Histology and Morphology

All grafts were perfused with sodium nitroprusside (10^{-5} mol/L) as a vasodilator and fixed with paraformaldehyde under 180 cm H_2O before embedding in paraffin. The grafts were cut transversely into 3 blocks, fixed in 4% phosphate-buffered paraformaldehyde, 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, was measured as described previously to examine the Rho-kinase activity in vivo.[15] We loaded an equal amount of protein on each well of polyacrylamide gel for the electrophoresis. The amount of proteins derived from vascular wall cells is different among samples because each sample, especially allografts, contains extracellular matrix (ECM). Therefore, each band intensity of the ERM was normalized by a corresponding value of total actin.[17] We used an antibody to phosphorylated ERM and that to total ERM that we developed ourselves[18] and rabbit anti-total actin antibody (A2066, Sigma, St Louis, Mo).

Isolation of RNA and Ribonuclease Protection Assay

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.

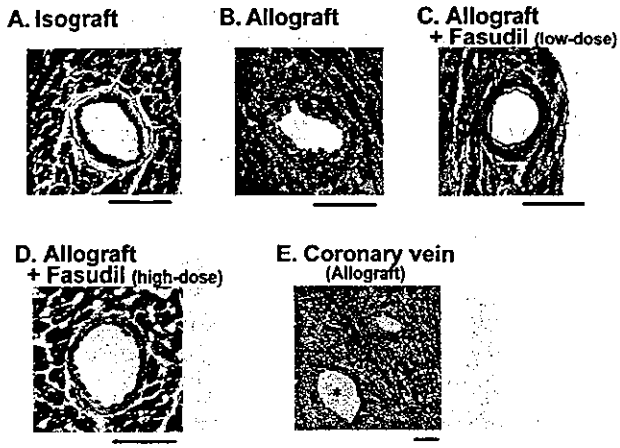

Figure 1. Representative photomicrographs of a mouse coronary artery from an isograft (A) and from an allograft in the control (B), the low-dose (C) and the high-dose fasudil group (D) at 4 weeks after the transplantation (Masson's trichrome staining, ×200). In the control allograft group, intimal thickening and perivascular fibrosis were noted, both of which were dose-dependently suppressed by fasudil treatment. Coronary vein, indicated by an asterisk, in the allograft is shown for comparison with coronary arteries (E). Bars=100 μm.

Immunohistochemistry

Four weeks after the transplantation, cardiac grafts were cut horizontally into 4 blocks, embedded in OTC compound (Sakura Finetechnical 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

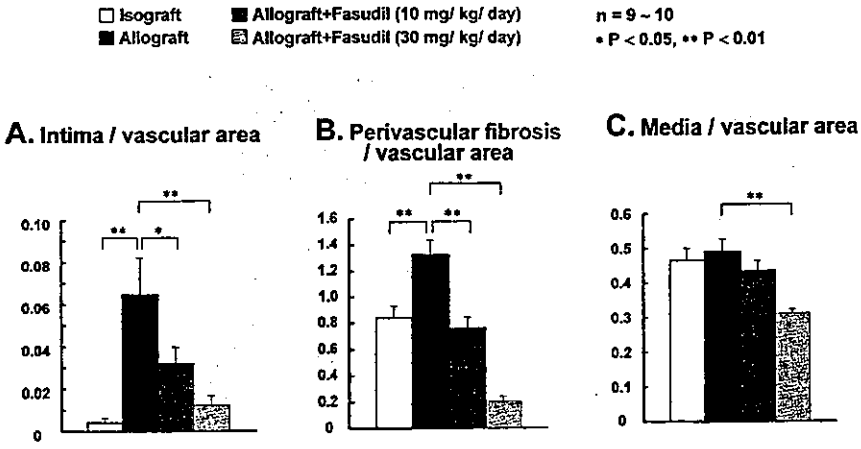

Figure 2. Long-term treatment with hydroxyfasudil inhibits the development of CAV in mice. In the control allograft group, intimal thickening (A, as expressed by intima/vascular area ratio) and perivascular fibrosis (B) developed at 4 weeks after the transplantation, both of which were dose-dependently suppressed by the fasudil treatment. By contrast, medial thickness was reduced only in the high-dose fasudil group (C).

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.

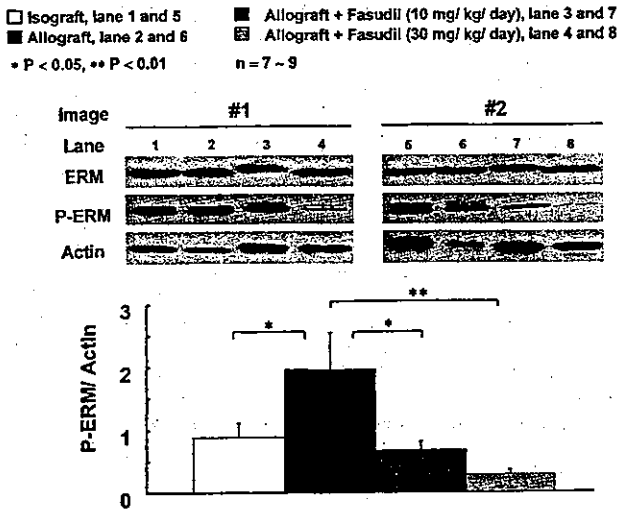

Figure 3. Representative images and quantified analysis of Western blotting for phosphorylated ERM, a marker of Rho-kinase activity, total ERM, and actin, in cardiac grafts at 4 weeks after cardiac transplantation. The Rho-kinase activity was increased in the control allograft group, which was dose-dependently suppressed by the fasudil treatment.

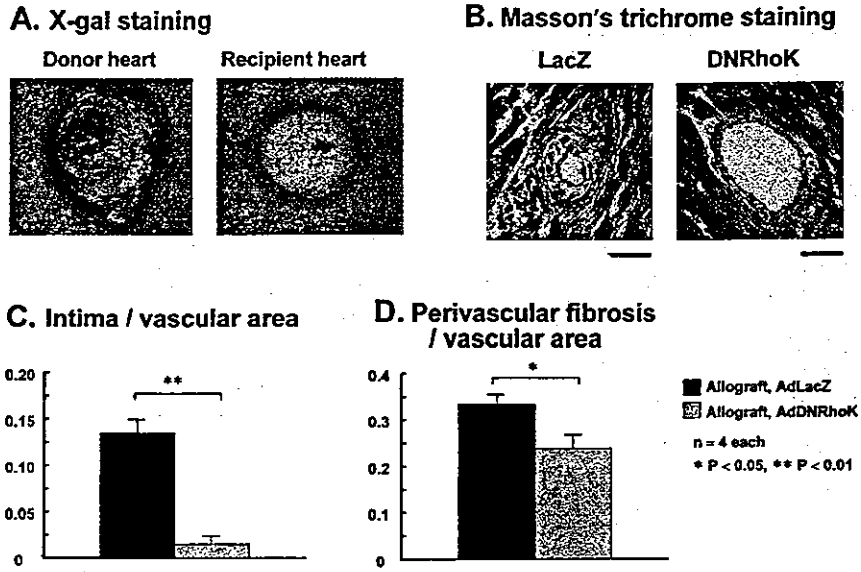

Figure 4. Inhibitory effects of in vivo gene transfer of DN-Rho-kinase on CAV in mice. DN-Rho-kinase and LacZ were transfected into allografts immediately after transplantation. A, X-gal staining performed 1 week after the transfection confirmed the expression of LacZ. B, Representative photomicrographs of a coronary artery in the allograft groups transfected with LacZ or DN-Rho-kinase. The intimal thickening (as expressed by the intima/vascular area ratio) (C) and perivascular fibrosis/vascular area ratio (D) were suppressed by in vivo gene transfer of DN-Rho-kinase. Bars=100 μm.

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.[20–22] The inhibitory effect of hydroxyfasudil (≈90%), at safe doses,[23] 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.[24] 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,[12,14] 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-

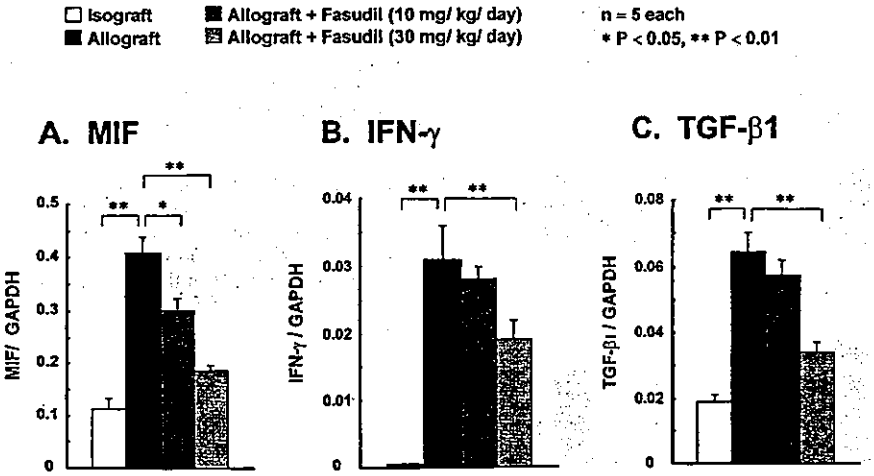

Figure 5. Inhibitory effects of the fasudil treatment on mRNA expression for MIF (A), IFN-γ (B), and TGF-β1 (C) in cardiac allograft at 2 weeks after the transplantation (RNase protection assay). In the control allograft group, the mRNA expressions for the 3 cytokines were all increased and were dose-dependently suppressed by the fasudil treatment.

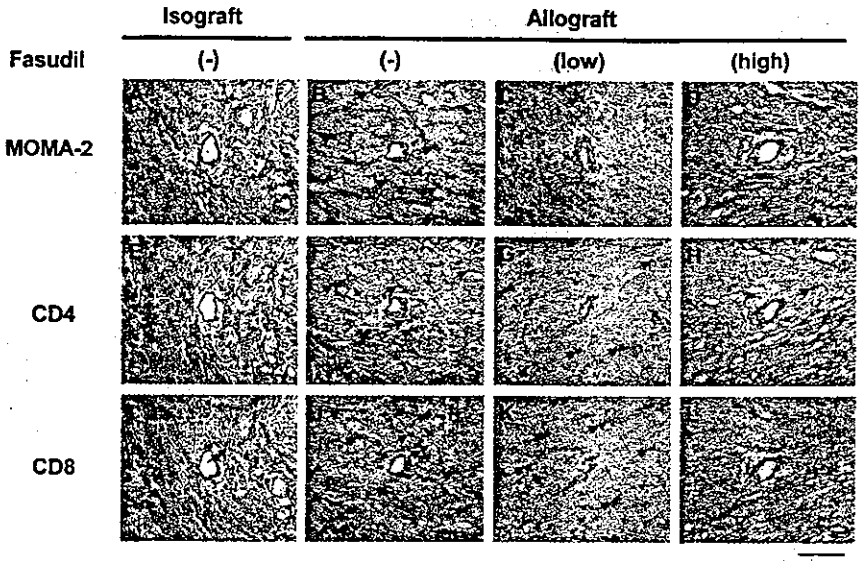

Figure 6. Representative photomicrographs of the mouse heart from the isograft group (A, E, and I) and from the allograft groups without (B, F, and J) and with a low dose (C, G, and K) and a high dose (D, H, and L) of fasudil at 4 weeks after the cardiac transplantation (×200). The accumulation of macrophages (MOMA-2) and CD4- and CD8-positive T cells, which are shown by arrows, was dose-dependently suppressed by the fasudil treatment. Bar=100 μm.

term CAV was developed using an H-2 identical combination, AKR (H-2^k) to C3H (H-2^k).[11,25] 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.[25] Since the coronary vascular lesions in this model have many similarities to those in humans,[25,26] the model has been used to examine the pathogenesis of CAV.[27,28]

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.[29] 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.[30] 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.

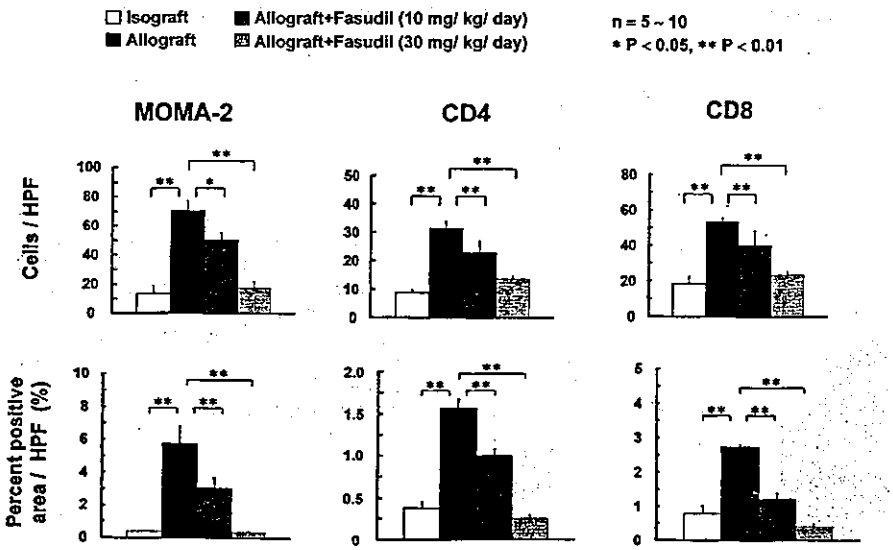

Figure 7. Quantitative analysis of the inhibitory effects of fasudil on the inflammatory cell accumulation in a cardiac allograft at 4 weeks after the transplantation in mice. The long-term treatment with hydroxyfasudil dose-dependently inhibited the accumulation of macrophages (MOMA-2) and CD4- and CD8-positive T cells. Top and bottom panels represent a number of those cells per high-power field (HPF) and a percent-positive area by those cells per HPF, respectively.

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 neointima as does Y-27632, another specific Rho-kinase inhibitor.[30,31] In addition, cytoplasmic translocation of ERM may be involved in an early phase of apoptosis.[32] Second, hydroxyfasudil could inhibit cell migration.[17,30,33] Third, hydroxyfasudil could suppress VSMC proliferation.[6]

Rho-kinase plays an important role in the pathogenesis of arteriosclerosis in vivo.[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[34] or in vivo gene transfer of DN-Rho-kinase[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-γ, and TGF-β1 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 plasminogen activator inhibitor-1 (PAI-1) but not extracellular signal–regulated protein kinase.[35]

MIF may be involved in the pathogenesis of graft rejection[36] and atherosclerosis.[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[35] and the downregulation of eNOS.[10] It has been reported that MIF upregulates the expression of ICAM-1 on endothelial cells while it decreases redox- or stress-induced apoptosis.[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-γ also may be involved in the progression of CAV. The development of CAV is suppressed in the grafts from IFN-γ–deficient mice, suggesting an involvement of the cytokine in the pathogenesis of the disorder.[38,39] TGF-β1 is known to increase fibronectin and type 1 collagen expression by fibroblasts.[40] In addition, an increased expression of fibronectin and laminin in the early posttransplantation period precedes cellular infiltration.[41,42] Thus, it is also conceivable that Rho-kinase–mediated upregulation of TGF-β1 is involved in the pathogenesis of CAV. In the present study, the expression of TNF-α, 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.[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.

Acknowledgments

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Long-Term Treatment With a Rho-Kinase Inhibitor Improves Monocrotaline-Induced Fatal Pulmonary Hypertension in Rats

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Abstract—Primary pulmonary hypertension is a fatal disease characterized by endothelial dysfunction, hypercontraction and proliferation of vascular smooth muscle cells (VSMCs), and migration of inflammatory cells, for which no satisfactory treatment has yet been developed. We have recently demonstrated that intracellular signaling pathway mediated by Rho-kinase, an effector of the small GTPase Rho, is involved in the pathogenesis of arteriosclerosis. In the present study, we examined whether the Rho-kinase–mediated pathway is also involved in the pathogenesis of fatal pulmonary hypertension in rats. Animals received a subcutaneous injection of monocrotaline, which resulted in the development of severe pulmonary hypertension, right ventricular hypertrophy, and pulmonary vascular lesions in 3 weeks associated with subsequent high mortality rate. The long-term blockade of Rho-kinase with fasudil, which is metabolized to a specific Rho-kinase inhibitor hydroxyfasudil after oral administration, markedly improved survival when started concomitantly with monocrotaline and even when started after development of pulmonary hypertension. The fasudil treatment improved pulmonary hypertension, right ventricular hypertrophy, and pulmonary vascular lesions with suppression of VSMC proliferation and macrophage infiltration, enhanced VSMC apoptosis, and amelioration of endothelial dysfunction and VSMC hypercontraction. These results indicate that Rho-kinase–mediated pathway is substantially involved in the pathogenesis of pulmonary hypertension, suggesting that the molecule could be a novel therapeutic target for the fatal disorder. (*Circ Res.* **2004;94:385-393.**)

Key Words: pulmonary hypertension ■ Rho-kinase ■ vascular smooth muscle cells ■ endothelial nitric oxide synthase ■ macrophages

Primary pulmonary hypertension (PPH) is a life-threatening disease characterized by a marked and sustained elevation of pulmonary artery pressure. The disease has no obvious causes and ultimately results in right ventricular (RV) failure and death. The pathological changes of hypertensive pulmonary arteries include endothelial injury, proliferation and hypercontraction of vascular smooth muscle cells (VSMCs), and migration of macrophages.[1-3] PPH continues to be a serious clinical problem with high morbidity and mortality.[4]

In 1990s, Rho-kinase/ROK/ROCK was identified as an effector of the small GTPase Rho,[5-7] which plays an important role in various cellular functions, including smooth muscle contraction, actin cytoskeleton organization, cell adhesion and motility, cytokinesis, and gene expression.[8-10] In a series of experimental and clinical studies, we have demonstrated that Rho-kinase–mediated pathway is substantially involved in the pathogenesis of arteriosclerosis.[11-17] These Rho-kinase–mediated alterations in blood vessels also may be involved in the pathogenesis of pulmonary hypertension (PH). In this study, we examined whether Rho-kinase–mediated pathway is involved in the pathogenesis of rat model of fatal PH in vivo.

Materials and Methods

The present study was approved by the Institutional Animal Care and Use Committee of the Kyushu University Graduate School of Medical Sciences.

Animal Model of Fatal PH

A total of 323 adult male Sprague-Dawley rats (Charles River, Yokohama, Japan; 250 to 300 g body weight) were used, including 156 for survival study, 83 for hemodynamic and histology study, 36

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