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PRECLINICAL RESEARCH

Beneficial Effect of Hydroxyfasudil, a Specific Rho-Kinase Inhibitor, on Ischemia/Reperfusion Injury in Canine Coronary Microcirculation In Vivo

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OBJECTIVES We examined whether hydroxyfasudil, a specific Rho-kinase inhibitor, exerts cardioprotective effect on coronary ischemia/reperfusion (I/R) injury and, if so, whether nitric oxide (NO) is involved.

BACKGROUND Recent studies have demonstrated that Rho-kinase is substantially involved in the pathogenesis of cardiovascular diseases; however, it remains to be examined whether it is also involved in ischemia/reperfusion (I/R) injury.

METHODS Canine subepicardial small arteries (SA, ≥100 μm) and arterioles (A, <100 μm) were observed by a charge-coupled device intravital microscope during I/R. Coronary vascular responses to endothelium-dependent (acetylcholine, intracoronary [IC]) and -independent (papaverine, IC) vasodilators were examined after I/R under the following four conditions: control (n = 7), NO synthase inhibitor alone (N^G-monomethl-L-arginine [L-NMMA], IC, n = 4), hydroxyfasudil alone (IC, n = 7), and hydroxyfasudil plus L-NMMA (n = 7).

RESULTS Hydroxyfasudil significantly attenuated serotonin (IC)-induced vasoconstriction of SA (−7 ± 1% vs. 2 ± 1%, $p < 0.01$). Coronary I/R significantly impaired coronary vasodilation to acetylcholine after I/R (SA, $p < 0.05$; and A, $p < 0.01$ vs. before I/R) and L-NMMA further reduced the vasodilation, whereas hydroxyfasudil completely preserved the responses. The vasoconstriction by L-NMMA after I/R was significantly improved by hydroxyfasudil in both-sized arteries (both $p < 0.01$). Expression of endothelial nitric oxide synthase (eNOS) protein in the ischemic endocardium of left anterior descending coronary artery area (as determined by Western blotting) significantly decreased (79 ± 4%) compared with the nonischemic endocardium of LCX area (100 ± 7%), which was improved by hydroxyfasudil (105 ± 6%, $p < 0.01$). Hydroxyfasudil significantly reduced myocardial infarct size, and hydroxyfasudil with L-NMMA also reduced the infarct size compared with L-NMMA alone.

CONCLUSIONS Hydroxyfasudil exerts cardioprotective effects on coronary I/R injury in vivo, in which NO-mediated mechanism may be involved through preservation of eNOS expression. (J Am Coll Cardiol 2005;45:599–607) © 2005 by the American College of Cardiology Foundation

Ischemia-reperfusion (I/R) injury attenuates endothelium-dependent dilation of large coronary arteries both in vitro (1,2) and in vivo (3,4). Endothelial dysfunction causes adverse outcome in the coronary circulation (5). Reperfusion injury is caused by direct myocardial injury through coronary vasospasm, free radicals, and inflammatory responses (6,7). Furthermore, local coronary vasoconstrictions in response to vasoconstrictors (e.g., serotonin) are enhanced (8,9). However, the mechanism of I/R-induced vascular injury remains to be clarified.

Recent studies have demonstrated that Rho-kinase, an effector of the small guanosine triphosphatase Rho, is substantially involved in the pathogenesis of cardiovascular diseases (10). Shimokawa et al. (10,11) have recently found that hydroxyfasudil is a potent and specific inhibitor of Rho-kinase and markedly inhibits coronary hyperconstriction and macrophage migration. They also demonstrated that intracoronary serotonin induces coronary hypercontractions at the inflammatory coronary lesions both in vitro and in vivo, in which up-regulated Rho-kinase is substantially

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Abbreviations and Acronyms
I/R = ischemia-reperfusion
LAD = left anterior descending coronary artery
LCX = left circumflex artery
NO = nitric oxide

involved (12). Recent studies demonstrated that endothelial expression and activity of Rho-kinase are enhanced by hypoxia, with a resultant down-regulation of endothelial nitric oxide synthase (eNOS) expression and reduced nitric oxide (NO) production (13), and that Rho-kinase is also involved in a canine model of cerebral infarction associated with superoxide production and neutrophil infiltration (14).

It is conceivable that Rho-kinase is involved in the mechanisms of I/R injury associated with reduced endothelial NO production. In this study, we thus examined whether hydroxyfasudil exerts protective effect on coronary I/R injury in vivo and, if so, whether NO is involved.

METHODS

Animal preparation. This study conformed to the Guideline on Animal Experiments of Kawasaki Medical School and the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

Mongrel dogs (15 to 25 kg, n = 31) of either gender were anesthetized with morphine (3 mg/kg, intramuscular) and sodium pentobarbital (25 mg/kg, intravenous). After intubation, each animal was ventilated with a high-frequency jet ventilator (model VS600, IDC, Pittsburgh, Pennsylvania) with room air supplemented by 100% oxygen. Aortic pressure and left ventricular pressure were continuously monitored with an 8-F pigtail double manometer catheter (SPC-784A, Millar, Texas). The proximal portion of the left anterior descending coronary artery (LAD) was isolated and a transonic flow probe (T206, Transonic Systems, Ithaca, New York) was placed around the vessel.

Needle-probe intravital microscope. The needle-probe (4.5 mm in diameter, VMS 1210, Nihon Kohden, Tokyo, Japan) contains a gradient index lens (with a magnification of 200) surrounded by light guide fibers and a double lumen sheath. A doughnut-shaped balloon on the tip avoids direct compression of the vessels by the needle tip (15).

Measurements of coronary diameters. We placed the needle probe gently on subepicardial microvessels. When a clear vascular image was obtained, end-diastolic vascular images were taken with 30 pictures/s (15).

Measurements of regional myocardial blood flow. Regional myocardial blood flow was determined by the nonradioactive microsphere (Sekisui Plastic Co, Ltd, Tokyo, Japan) technique, as previously described in detail (16). Briefly, 1 ml of the microspheres suspension (2 to 4 × 10^6 spheres) was injected into the left atrium 85 min after the onset of coronary occlusion. Just before microsphere administration, a reference blood flow sample was drawn from the femoral artery at a constant rate of 8 ml/min for 2 min. The X-ray fluorescence of the stable heavy elements was measured by a wavelength-dispersive spectrometer (model PW 1480, Phillips Co., Ltd., Eindhoven, the Netherlands) (16). Myocardial blood flow was calculated according to the formula: time flow = tissue counts × (reference flow/reference counts) and was expressed in ml/g per minute (16).

Western blotting. Proteins were separated on sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis as previously described (17). The tissues were homogenized in a sample buffer (100 mM Tris-HCl [pH 6.8], 4% SDS, 0.2% glycerol). The tissue lysate was centrifuged and the supernatant collected. Protein concentration was quantified by a bicinchoninate (BCA) protein assay kit (Pierce Chemical, Rockford, Illinois). An aliquot of 10 μg of protein from each sample was electrophoresed on a 7.5% SDS-polyacrylamide gel. Proteins were subsequently transferred to polyvinylidine difluoride membrane (Immobilon-P membrane, Millipore, Bedford, Massachusetts) electrophoretically (100 V for 1 h) and membranes were incubated with antibody. The antibodies used in this study were rabbit anti-phosphorylated ezrin/radixin/moesin (ERM) family, total ERM. The antibody against phosphorylated ERM recognizes human moesin (phosphorylated at Thr558), which also binds to the phosphorylated ezrin (Thr567) and radixin (Thr564). Therefore, we used the extent of phosphorylation of ERM as a marker of Rho-kinase activity. The levels of Western blot for phosphorylated ERM were normalized to those for total ERM as a control. Membranes were then incubated with a horseradish peroxidase-conjugated horse anti-rabbit immunoglobulin G antibody (1:5,000). Immunoreactivity was detected by enhanced chemiluminescence autoradiography (ECL Western blotting detection kit; Amersham Pharmacia Biotechnology, United Kingdom).

The obtained samples were washed with ice-cold Tris-HCl buffer (pH 7.4), mixed with the sample buffer (4% sodium lauryl sulfate, 12% beta-mercaptoethanol, and 20% glycerol in 100 mM Tris-HCl [pH 6.8]), sonicated (1 min), boiled (3 min), and finally centrifuged (10,000 g, 60 min, 4°C). The resultant supernatant was stored at −80°C until use. The separation of proteins was carried out according to the previous study (18), with a minor modification. The relative intensity of immunoreactive bands was quantified by Image Master 1D Elite software (Amersham Biotech, Buckinghamshire, United Kingdom), and the data were estimated as percentage of each control.

Experimental protocols. After the surgical procedure and instrumentation, at least 30 min were allowed for stabilization while hemodynamic variables were monitored. The following protocols were examined.

1. We infused graded doses of hydroxyfasudil (10, 30, and 100 μg/kg, IC), and coronary vascular responses were

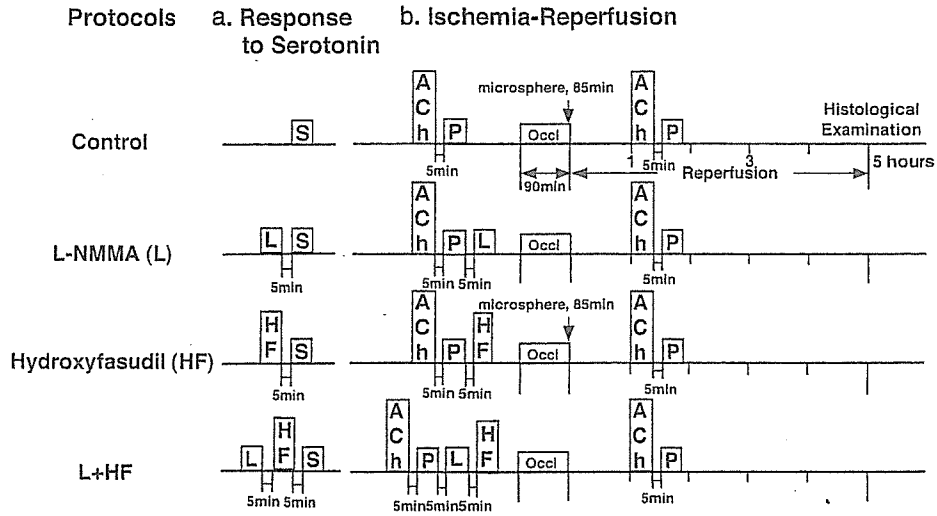

Figure 1. Experimental protocol. S = serotonin; L = L-NMMA; HF = hydroxyfasudil; Ach = acetylcholine; P = papaverine; Occl = coronary occlusion.

analyzed for 4 min by measuring end-diastolic vascular diameters and flows of the LAD.

2. The arteriolar vasoconstrictor response to serotonin before and after hydroxyfasudil (100 μg/kg, IC) was examined with or without inhibition of NO synthase (L-NMMA, 2 μmol/min for 20 min, IC) (Fig. 1). Hydroxyfasudil or L-NMMA was administered at 5 min before infusion of serotonin. The time interval between L-NMMA and hydroxyfasudil was also 5 min.
3. The arteriolar vasodilator responses to endothelium-dependent (acetylcholine, 1 μg/kg IC) and -independent (papaverine, 1 mg IC) vasodilators were examined before and after coronary I (90 min)/R (60 min) under the following four conditions separately in different animals: 1) control conditions, 2) L-NMMA alone, 3) hydroxyfasudil alone (100 μg/kg IC), and 4) hydroxyfasudil plus L-NMMA (Fig. 1). The time interval between each treatment was also 5 min. The basal coronary diameter is before administration of acetylcholine or papaverine either before or after I/R. Hydroxyfasudil and L-NMMA were administered at 5 min after administration of acetylcholine or papaverine. Microspheres were administered at 85 min after the onset of coronary occlusion.
4. After 5 h of reperfusion, LAD and the left circumflex artery (LCX) and myocardial tissue of LAD and LCX area were obtained for Western blotting. We reoccluded the LAD and injected Evans blue dye into a systemic vein. Then myocardial slices (5 mm) were incubated in 1% 2,3,5-triphenyltetrazolium chloride (Sigma, Japan) solution to detect the infarct area. Infarct size was expressed as percentage of the infarct area that was contiguous with area at risk (19).

Drugs. We used the following drugs: hydroxyfasudil (Asahi Kasei Pharma, Tokyo, Japan), acetylcholine (Daiichi-Seiyaku, Tokyo, Japan), papaverine (Dainihon-Seiyaku, Tokyo, Japan), and N^G-methyl-L-arginine (L-

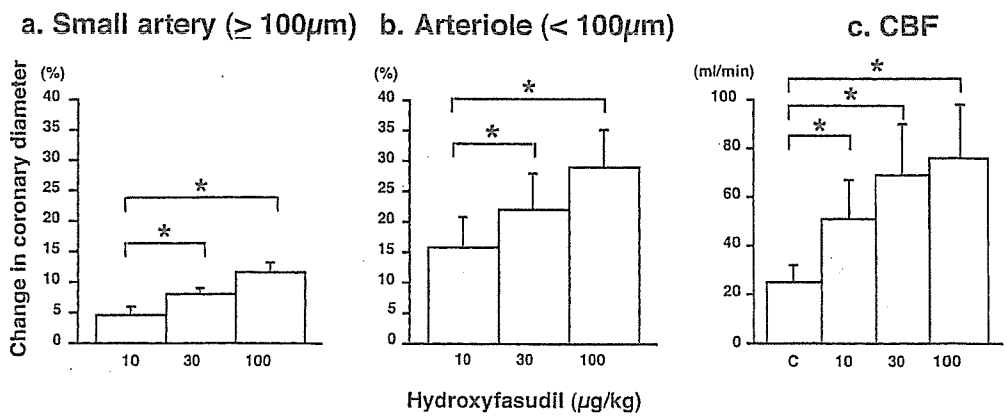

Figure 2. Coronary vasodilator effects of hydroxyfasudil in dogs in vivo. Hydroxyfasudil (10, 30, and 100 μg/kg, IC) caused coronary vasodilation, in a dose-dependent manner, under normal conditions in both small arteries (**a**) and arterioles (**b**). Number of vessels per animal used was 5/3 in small arteries and 7/4 in arterioles, respectively. Hydroxyfasudil also increased coronary blood flow (CBF) in a dose-dependent manner (**c**). *$p < 0.05$.

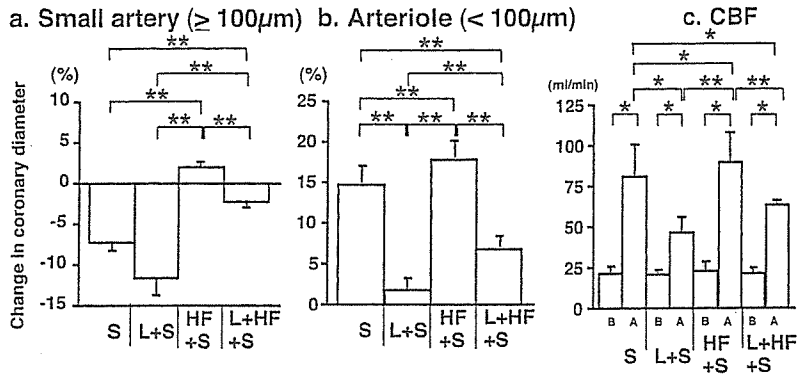

Figure 3. Effects of hydroxyfasudil on serotonin-induced coronary vascular responses in dogs in vivo. Hydroxyfasudil converted the serotonin-induced vasoconstriction of small arteries to vasodilation (a) and significantly enhanced the serotonin-induced vasodilation of arterioles (b). L-NMMA significantly attenuated the serotonin-induced vasodilation, which was counteracted by hydroxyfasudil. Number of vessels per animal used was 18/6 for S, L + S and HF + S, 13/4 for L + HF + S. *$p < 0.05$, **$p < 0.01$. S = serotonin; L = L-NMMA; HF = hydroxyfasudil; B = before drug; A = after drug.

NMMA, Sigma). All drugs were diluted in a physiologic saline immediately before use.

Statistical analysis. Results are expressed as means ± SEM. Vascular responses (Figs. 2a to 2c, 3c, 4c, 6c, 7a to 7c, 8a) were analyzed by one-way analysis of variance followed by Scheffe's post-hoc test for multiple comparisons. Difference in the effects of serotonin, acetylcholine, and papaverine on subepicardial microvessels before and after I/R (Figs. 3a, 3b, 4a, 4b, 5a to 5d, 6a, and 6b), and difference between infarct size/risk area and transmural collateral flow with or without hydroxyfasudil (Fig. 8b) were examined by a multiple regression analysis using a model in which the change in coronary diameter was set as a dependent variable (y) and vascular size as an explanatory variable (x) while the statuses of hydroxyfasudil and hydroxyfasudil plus L-NMMA were set as dummy variables (D_1, D_2) in the following equation; $y = a_0 + a_1x + a_2D_1 + a_3D_2$, where a_0 through a_3 are partial regression coefficients. The criterion for statistical significance was at $p < 0.05$.

RESULTS

Coronary vasodilator effects of hydroxyfasudil. Intracoronary administration of hydroxyfasudil caused a significant coronary vasodilation of both small arteries and arterioles (Figs. 2a and 2b, both $p < 0.05$, 10 μg/kg vs. 30 and 100 μg/kg) in a dose-dependent manner under control conditions with a resultant increase in CBF (Fig. 2c, $p < 0.05$, C vs. 10, 30 and 100 μg/kg). Intracoronary hydroxyfasudil did not significantly alter mean aortic pressure or heart rate (Table 1).

Hemodynamics and blood gases during I/R injury. In each experimental condition, mean aortic pressure and heart rate at baseline were constant and comparable (Table 1), and oxygen partial pressure (Po_2), carbon dioxide partial pressure (Pco_2), and pH were maintained within the physiologic ranges (pH 7.35 to 7.45, Pco_2 25 to 40 mm Hg, Po_2 >70 mm Hg) throughout the experiments. Hemodynamic

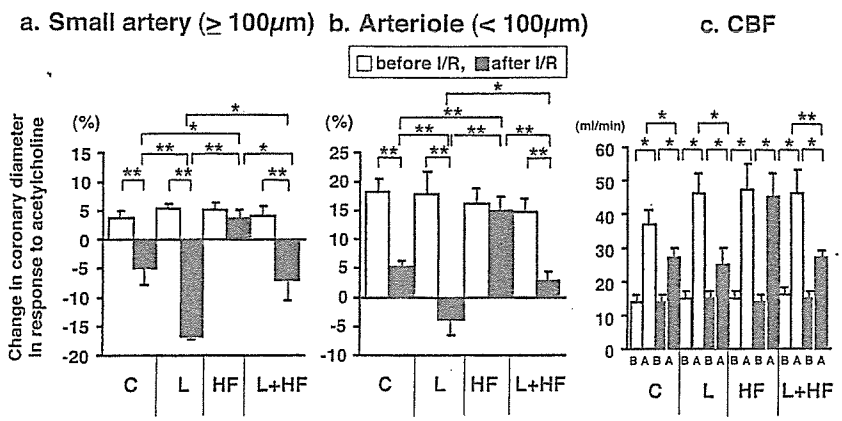

Figure 4. Endothelium-dependent coronary vasodilation before and after coronary ischemia/reperfusion (I/R) injury in dogs in vivo. Coronary I/R significantly impaired coronary vasodilation to acetylcholine under control conditions (C) and L-NMMA (L) further suppressed the vasodilation, whereas hydroxyfasudil (HF) completely preserved the responses. The vasoconstriction induced by L-NMMA after I/R was significantly improved by hydroxyfasudil in small arteries. Hydroxyfasudil also prevented the decrease in coronary blood flow (CBF) after I/R, which effect was attenuated by L-NMMA. Number of vessels per animals used was 7/6 for control (mean diameter 120 ± 7 μm), 5/4 for L-NMMA (123 ± 8 μm), 6/4 for hydroxyfasudil (118 ± 8 μm), and 5/4 for hydroxyfasudil plus L-NMMA (125 ± 9 μm) in small arteries, and 12/6 for control (70 ± 6 μm), 8/4 for L-NMMA (69 ± 7 μm), 8/5 for hydroxyfasudil (68 ± 7 μm), and 11/6 for hydroxyfasudil plus L-NMMA (71 ± 5 μm) in arterioles. I/R = ischemia/reperfusion; B = before acetylcholine; A = after acetylcholine. *$p < 0.05$; **$p < 0.01$.

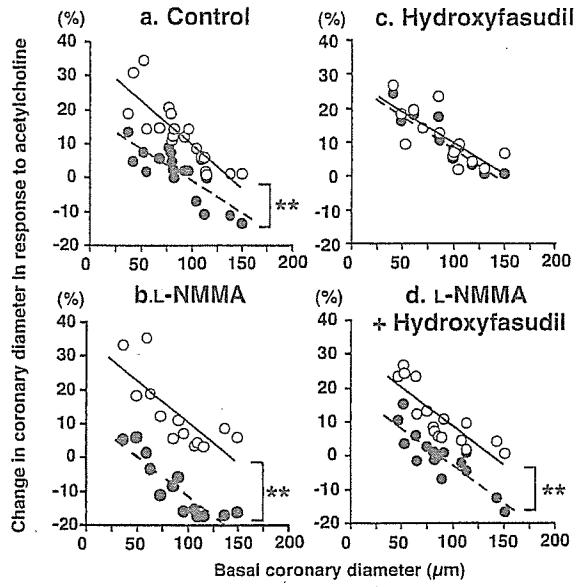

Figure 5. Coronary microvascular responses to acetylcholine before and after coronary ischemia/reperfusion (I/R) injury in dogs in vivo. Under control conditions, I/R significantly impaired coronary vasodilator response to acetylcholine **(a)**, whereas hydroxyfasudil completely preserved the responses in the absence **(c)** or presence of L-NMMA **(d)** compared with that in the presence of L-NMMA alone **(b)**. Number of vessels per animals used was 19/7 under control conditions (before I/R: $y = -0.3x + 35.9$, $r = 0.85$; after I/R: $y = -0.2x + 18.1$, $r = 0.80$), 13/4 for L-NMMA alone (before I/R: $y = -0.2x + 35.1$, $r = 0.76$; after I/R: $y = -0.2x + 12.2$, $r = 0.88$), 14/7 for hydroxyfasudil (before I/R: $y = -0.2x + 27.9$, $r = 0.73$; after I/R: $y = -0.2x + 27.4$, $r = 0.80$), and 16/7 for hydroxyfasudil plus L-NMMA (before I/R: $y = -0.2x + 31.8$, $r = 0.83$; after I/R: $y = -0.2x + 19.2$, $r = 0.86$). **$p < 0.01$. **Open circles** = before I/R; **solid circles** = after I/R.

variables at baseline did not significantly change after I/R compared with those before I/R (Table 1).

Effects of Rho-kinase inhibition on serotonin-induced coronary responses. Intracoronary administration of serotonin caused coronary vasoconstriction of small arteries and vasodilation of arterioles under control conditions (Figs. 3a and 3b, both $p < 0.01$ vs. basal coronary diameter). Intracoronary administration of L-NMMA enhanced the serotonin-induced vasoconstriction and abolished the serotonin-induced vasodilation of arterioles (Fig. 3b, $p < 0.01$ vs. serotonin, S). By contrast, hydroxyfasudil reversed the serotonin-induced vasoconstriction of small arteries to vasodilation while it further enhanced the serotonin-induced vasodilation of arterioles (Figs. 3a and 3b, both $p < 0.01$). The vasodilator effect of hydroxyfasudil on the coronary response to serotonin was significantly attenuated by L-NMMA in both-sized arteries (Figs. 3a and 3b, both $p < 0.01$). As a result, serotonin-induced increase in CBF (Fig. 3c) was significantly inhibited by L-NMMA ($p < 0.05$) and enhanced by hydroxyfasudil ($p < 0.05$), the effect of which was significantly attenuated by L-NMMA ($p < 0.01$).

Endothelium-dependent coronary vasodilation before and after I/R. Under control conditions (before I/R), intracoronary administration of acetylcholine caused a significant coronary vasodilation to a greater extent in arterioles than in small arteries (Figs. 4a, 4b, and 5a, $p < 0.01$). Coronary I/R significantly impaired the coronary vasodilation to acetylcholine in both sized arteries (both $p < 0.01$) and L-NMMA further reduced the vasodilation (Figs. 4a, 4b, and 5b both $p < 0.01$), whereas hydroxyfasudil completely preserved (small artery $p < 0.05$, arteriole $p < 0.01$) the acetylcholine-induced coronary vasodilator response after I/R (Figs. 4a and 4b). The vasoconstriction by L-NMMA was significantly attenuated by hydroxyfasudil in both sized arteries (both $p < 0.05$) with decrement of CBF (Figs. 4a to 4c). When the coronary vasodilator response to acetylcholine was expressed as a function of basal coronary diameter, hydroxyfasudil preserved the response after I/R injury at all sized coronary arteries either in the absence

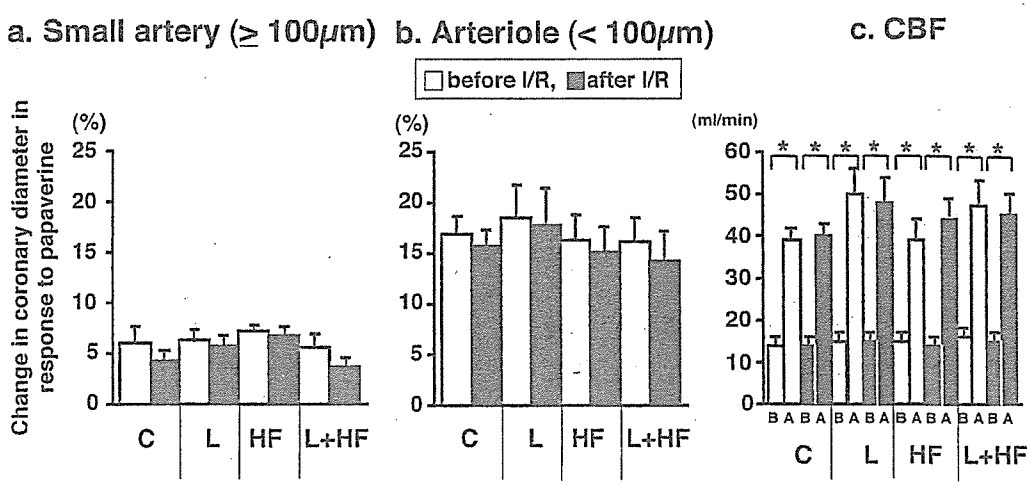

Figure 6. Endothelium-independent coronary vasodilation before and after coronary I/R injury in dogs in vivo. Coronary vasodilator response to papaverine was comparable under all conditions in both small arteries and arterioles. Number of vessels per animals used was 7/6 for control (mean diameter 120 ± 7 μm), 5/4 for L-NMMA (123 ± 8 μm), 6/4 for hydroxyfasudil (118 ± 8 μm), and 5/4 for hydroxyfasudil plus L-NMMA (125 ± 9 μm) in small arteries; and 12/6 for control (70 ± 6 μm), 8/4 for L-NMMA (69 ± 7 μm), 8/5 for hydroxyfasudil (68 ± 7 μm), and 11/6 for hydroxyfasudil plus L-NMMA (71 ± 5 μm) in arterioles. C = control; L = L-NMMA; HF = hydroxyfasudil. I/R = ischemia/reperfusion. B = before papaverine; A = after papaverine.

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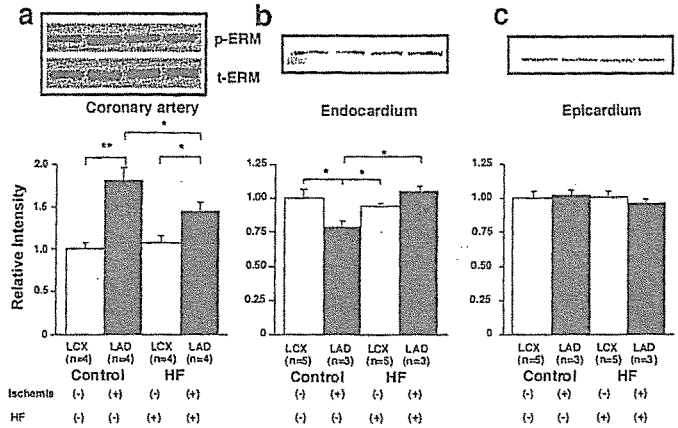

Figure 7. Western blotting showing the effects of hydroxyfasudil (HF) on Rho-kinase activity and on eNOS protein expression in the myocardium of LAD and LCX. **(a)** Rho-kinase activity in coronary artery; **(b)** expression of eNOS protein in endocardium; **(c)** expression of eNOS protein in epicardium. Rho-kinase activity was determined by the degree of ezrin-radixin-moesin phosphorylation (p-ERM/t-ERM). Rho-kinase activation in the ischemic LAD was completely inhibited by cotreatment with hydroxyfasudil. Expression of eNOS protein in the ischemic endocardium of LAD area was significantly decreased compared with the non-ischemic endocardium of LCX area, which was again improved by hydroxyfasudil. $^{*}p < 0.05$, $^{**}p < 0.01$.

(Figs. 4a, 4b, and 5c, df 2, 25, $p < 0.01$) or presence (Figs. 4a, 4b, and 5d, df 2, 24, $p < 0.01$) of L-NMMA compared with that in the presence of L-NMMA alone (Figs. 4a, 4b, and 5b).

Endothelium-independent coronary vasodilation. Coronary vasodilator response to papaverine was comparable under all conditions in both small arteries and arterioles (Figs. 6a and 6b). Similarly, the increase in CBF to papaverine (Fig. 6c) was also comparable under all conditions in both-sized arteries. Those coronary vasodilator responses were resistant to the blockade of NO synthesis with L-NMMA (Figs. 6a and 6b).

Activation of Rho-kinase by ischemia-reperfusion causes down-regulation of eNOS protein expression. Rho-kinase activity after a 90-min period of ischemia was significantly greater in the ischemic LAD than in the nonischemic LCX in the control group (Fig. 7a, $p < 0.01$). This Rho-kinase activation was significantly suppressed by hydroxyfasudil in the ischemic LAD (Fig. 7a, $p < 0.01$). Expression of eNOS protein in the ischemic endocardium of the LAD area (as determined by Western blotting) was significantly decreased (79 ± 4%, $p < 0.05$) compared with the nonischemic endocardium of the LCX area (100 ± 7%), which was also improved by hydroxyfasudil (105 ± 6%) (Fig. 7b, $p < 0.05$). There was no significant difference in the eNOS expression in the epicardium between the LAD and LCX area (Fig. 7c).

Effect of Rho-kinase inhibition on I/R-induced myocardial infarct size. Ischemia-reperfusion injury caused myocardial infarct area that was approximately 50% of the left ventricular risk area, and intracoronary L-NMMA did not further increase the I/R-induced infarction size (Fig. 8a). Intracoronary pretreatment with hydroxyfasudil markedly reduced the infarct size ($p < 0.01$ vs. control), and this beneficial effect of hydroxyfasudil was significantly attenuated by L-NMMA (Fig. 8a $p < 0.01$). In the control group, there was an inverse relation between the infarct area and collateral blood flow measured by microsphere technique ($r = 0.93$, $p < 0.01$), and hydroxyfasudil significantly shifted the regression line downward as compared with the control group ($p < 0.01$), that is, smaller infarct size for a given collateral flow (Fig. 8b).

DISCUSSION

The major findings of the present in vivo study in the canine coronary microcirculation were that: 1) a specific Rho-kinase inhibitor hydroxyfasudil preserved the endothelium-dependent coronary vasodilator responses after coronary I/R injury, 2) hydroxyfasudil also reduced myocardial infarct size, and 3) NO may be involved in those cardiovascular protective effects of hydroxyfasudil. To the best of our knowledge, this is the first report that demonstrates the usefulness of a Rho-kinase inhibitor to prevent coronary I/R injury in vivo.

Validations of experimental model and methodology. On the basis of the previous reports (4,12,20), we chose the adequate dose of hydroxyfasudil, acetylcholine, papaverine, and L-NMMA to examine the effects of the Rho-kinase inhibition, endothelium-dependent and -independent vasodilator responses, and inhibition of NO synthesis on coronary vascular responses before and after coronary I/R, respectively. The methodologic validity of the present study has been confirmed previously (15). After 60 to 90 min of ischemia, ultrastructural damage of coronary endothelium was observed particularly in the subendocardium in the present study, a consistent finding to the previous study (21).

Hydroxyfasudil as a specific Rho-kinase inhibitor in the coronary microcirculation in vivo. Shimokawa et al. (11) have recently demonstrated that hydroxyfasudil is a specific Rho-kinase inhibitor that markedly inhibits coronary vasospastic responses in a porcine model; its inhibitory effect on Rho-kinase is 100 times greater than on protein kinase C and

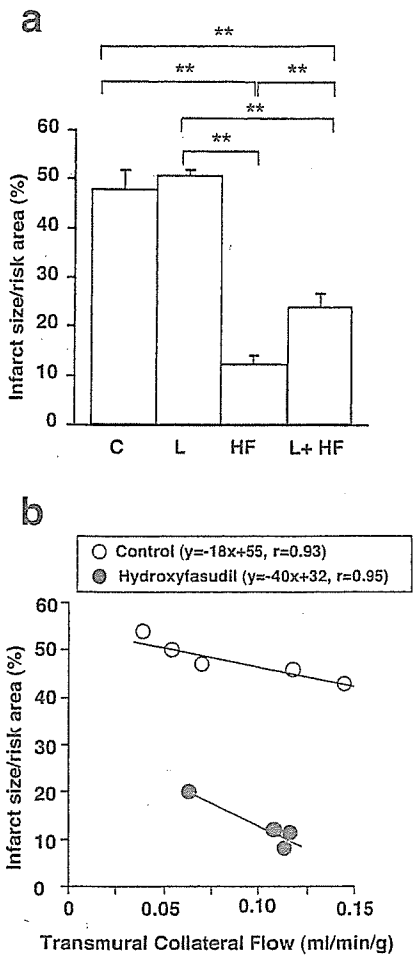

Figure 8. **(a)** Ischemia/reperfusion (I/R)-induced LV infarct size in dogs in vivo. Hydroxyfasudil significantly reduced the I/R-induced LV infarct size. The beneficial effect of hydroxyfasudil was partially attenuated by L-NMMA, while L-NMMA alone did not significantly increase the infarct size. Number of animals used was each 7 for C, HF, and L + HF, and 4 for L. C = control; L = L-NMMA; HF = hydroxyfasudil. $^{**}p < 0.01$. **(b)** Plot of infarct size expressed as a percentage of the risk area and regional collateral flow during ischemia. In the control group, there was an inverse relation between infarct area and collateral flow measured by microsphere ($r = 0.93$, $p < 0.01$), and hydroxyfasudil significantly shifted the regression line downward as compared with the control group ($p < 0.01$). Number of animals used was five for control conditions and four for hydroxyfasudil.

1,000 times greater on myosin light-chain kinase. Hydroxyfasudil has potent vasodilator effects on coronary arteries through inhibition of Rho-kinase-mediated phosphorylations of myosin light chains (11). In the present study, intracoronary hydroxyfasudil caused coronary microvascular vasodilation in a dose-dependent manner in vivo, and its vasodilator effect was greater in arterioles than in small arteries (Fig. 2). Hydroxyfasudil suppressed the serotonin-induced vasoconstriction of small arteries, whereas it enhanced the serotonin-induced vasodilation of arterioles in vivo (Fig. 3). This finding is in accordance with the hypothesis that the calcium sensitization of vascular smooth-muscle cells mediated by Rho-kinase plays a key role in the molecular mechanisms of coronary hyperconstriction (12). Furthermore, in the present study, intracoronary L-NMMA significantly attenuated serotonin-induced coronary vasodilator responses, which were improved by hydroxyfasudil, indicating an involvement of NO-mediated mechanism in the beneficial effects of the Rho-kinase inhibitor. Lamping et al. (22) demonstrated that coronary vascular response to serotonin is determined by a balance between 5-HT_1 receptor-mediated dilatation of coronary arterioles and 5-HT_2 receptor-mediated vasoconstriction of small coronary arteries. Inhibition of NO synthase enhances coronary vasoconstriction to serotonin in both-sized arteries. Our present results are in agreement with those of Lamping et al. The beneficial vasodilator effect of hydroxyfasudil on coronary vascular response to serotonin is mediated by its action on both vascular smooth muscle and the endothelium as shown in Figure 3. Thus, it is possible that the beneficial effect of Rho-kinase blockade with hydroxyfasudil is mediated by its action on both vascular smooth muscle and the endothelium (22). Serotonin released by aggregating platelets has been implicated for coronary vasospasm in the presence of damaged vascular endothelium (5,23).

Beneficial effects of a Rho-kinase inhibitor on coronary I/R injury. In the present study, hydroxyfasudil exerted beneficial effects on I/R-induced endothelial injury in the canine coronary microcirculation in vivo through the NO-dependent mechanism (Figs. 4 and 5). This dose of hydroxyfasudil (100 μg/kg) selectively inhibits Rho-kinase activity and effectively prevents serotonin-induced coronary hyperconstriction. Recent studies have demonstrated that cGMP-dependent protein kinase inhibits RhoA phosphorylation by inhibiting the membrane binding of RhoA, in which the NO-mediated mechanism may inhibit the RhoA/Rho-kinase pathway (24–26). It was previously demonstrated that statins attenuate I/R injury of the heart and the brain in rats and mice, demonstrating the Rho-mediated and NO-dependent protective effect of statins (27,28). Hydroxyfasudil also inhibits the production of superoxide anions in neutrophils (29) and various chemoattractant-induced migration of those cells (14) in a canine model of cerebral ischemia. Furthermore, treatment with hydroxyfasudil in human saphenous vein endothelial cells reversed the hypoxia-induced decrease in eNOS activity as examined by the citrulline conversion assay and 4,5-diaminofluorescein diacetate fluorescence method (13). In the present study, I/R increased Rho-kinase activity, and hydroxyfasudil significantly inhibited the Rho-kinase activation. These findings suggest that NO is involved in the protective effect of hydroxyfasudil with an increase in eNOS activity and a decrease in Rho-kinase activity during reperfusion injury.

In the present study, the vasodilator effects of hydroxyfasudil were significantly attenuated by L-NMMA (Figs. 3 and 4). The eNOS expression was decreased in the ischemic area of the endocardium compared with that of the epicardium under control conditions, which was improved by hydroxyfasudil (Fig. 7). We have previously demonstrated that endocardial arteriolar dilation during reactive hyperemia is more sensitive to L-NMMA than epicardial arte-

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Table 1. Hemodynamics During Myocardial Ischemia-Reperfusion Injury in Dogs

		Before I/R			After I/R		
	n	Baseline	ACh	Papaverine	Baseline	ACh	Papaverine
MBP (mm Hg)							
Control	7	91 ± 4	90 ± 6	92 ± 5	89 ± 4	89 ± 5	92 ± 6
L-NMMA	4	88 ± 8	86 ± 5	91 ± 7	86 ± 4	86 ± 4	85 ± 4
Hydroxyfasudil	7	92 ± 9	92 ± 8	93 ± 8	93 ± 6	90 ± 6	91 ± 7
L-NMMA + hydroxyfasudil	7	89 ± 6	89 ± 5	89 ± 5	91 ± 8	87 ± 10	89 ± 9
Heart rate (beats/min)							
Control	7	151 ± 5	156 ± 3	155 ± 3	155 ± 5	153 ± 5	152 ± 5
L-NMMA	4	147 ± 7	149 ± 8	149 ± 8	145 ± 11	146 ± 11	145 ± 11
Hydroxyfasudil	7	152 ± 7	151 ± 8	148 ± 8	148 ± 7	149 ± 7	150 ± 7
L-NMMA + hydroxyfasudil	7	151 ± 6	152 ± 6	151 ± 6	154 ± 6	151 ± 6	153 ± 7

Results are expressed as mean ± SEM.
ACh = acetylcholine; I/R = ischemia/reperfusion; MBP = mean blood pressure.

riolar dilation (30). These findings indicate that the perfusion of the endocardium is more dependent on NO than that of the epicardium and that endothelial damage after I/R in arterioles may be greater in the endocardium than in the epicardium.

In the present study, hydroxyfasudil exerted cardiovascular protective effects on coronary I/R injury, as did preconditioning (31,32). However, the mechanism by which hydroxyfasudil and preconditioning protect coronary I/R injury appears to be different. Endogenous NO does not alter the infarct size after I/R and is not involved in the protective mechanism of preconditioning in pigs or rabbits (33,34). It has been suggested that preconditioning preserves myocardial creatine phosphate and intracellular pH (35). Furthermore, ischemic preconditioning increases adenosine production and activates protein kinase C, which also enhances adenosine production during I/R injury.

In the present study, hydroxyfasudil significantly reduced myocardial infarct size with increment of coronary collateral blood flow, at least in part, thorough the NO-mediated mechanism (Fig. 8). Shimokawa et al. (11) demonstrated that hydroxyfasudil inhibits both MLC mono- and diphosphorylations. Satoh et al. (14) showed that hydroxyfasudil also protects the brain from ischemic injury through inhibition of superoxide production and neutrophil infiltration. Mohri et al. (36) demonstrated that fasudil suppresses coronary microvascular spasm in patients with microvascular angina. Wolfrum et al. (37) recently demonstrated that inhibiting Rho-kinase has cardioprotective effects to reduce infarct size by activating phosphatidylinositol 3-kinase/protein kinase Akt/eNOS pathways. All these mechanisms may be involved in the beneficial effects of hydroxyfasudil on the I/R-induced myocardial injury.

Hydroxyfasudil increases blood supply to the ischemic region of the myocardium and prevents I/R-induced myocardial injury. Furthermore, it has been recently demonstrated that an estrogen receptor modulator, raloxifene, also reduces I/R-induced myocardial infarct size, whereas an inhibitor of NO synthesis (L-NAME) or a blocker of calcium-activated K^+ channels (charybdotoxin) partly attenuates the effect of raloxifene (19). These results suggest that cardioprotective effects of those inhibitors may be mediated in part by the compensatory effects of NO and endothelium-derived hyperpolarizing factor (20). Several studies using NO synthase inhibitors (38,39) or eNOS-deficient mice (40) demonstrated an increase in infarct size after I/R. The effect of NO synthesis inhibition on the infarct size might be species- and dose-dependent.

Clinical implications and conclusions. The present study has demonstrated for the first time that hydroxyfasudil, a specific Rho-kinase inhibitor, has NO-dependent cardiovascular protective effects on coronary I/R injury in vivo. Rho-kinase inhibitor has also an antianginal effect in a canine model of angina (41), patients with effort angina (42), and those with vasospastic angina (43). Moreover, it has been recently reported that hydroxyfasudil may be effective for the treatment of pulmonary hypertension (44). Indeed, Rho-kinase inhibitors may be useful for the treatment of a wide range of cardiovascular diseases (10). The present study suggests that Rho-kinase inhibitors may also be useful for the treatment of coronary I/R injury in humans.

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Original Article

Dilated cardiomyopathy after pacemaker implantation in complete heart block

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Abstract

Background: The aim of this study was to evaluate the clinical features of patients with congenital complete heart block (CCHB) who developed dilated cardiomyopathy (DCM) after pacemaker implantation (PMI) and to determine factors predicting DCM development.

Method: A total of 15 patients were reviewed retrospectively. They were classified into two groups, one consisted of four patients who were diagnosed as having CCHB *in utero* or at birth and who developed DCM after PMI (DCM group) and the other consisted of 11 patients who did not (non-DCM group).

Results: Maternal autoantibodies were found in two of the DCM group and in five of the non-DCM group. Perfusion defects in myocardial imaging were detected in all DCM patients and in five non-DCM patients. DCM developed 2 to 43 months after PMI and three DCM patients died of heart failure 7 to 48 months after PMI. In pathological studies, endocardial or interstitial fibrosis was present in all DCM patients and in one of two in the non-DCM group. No significant differences between the two groups were found in age at PMI, atrial or ventricular rate, end-diastolic dimension and ejection fraction of the left ventricle before PMI, and width of QRS after PMI.

Conclusion: Although it was suspected that the patients with CCHB had myocardial involvement before PMI, there was no significant factor predicting the risk of DCM after PMI. In addition to cardiac rhythm abnormalities, careful attention should be paid to cardiac function in CCHB patients after PMI.

Key words complete heart block, dilated cardiomyopathy, pacemaker implantation.

Congenital complete heart block (CCHB) without intracardiac structural abnormalities is potentially lethal, however, with effective management during the prenatal, neonatal and infantile periods, and prompt initiation of cardiac pacing, the prognosis is considered benign.[1,2] Nevertheless, despite appropriate initiation of cardiac pacing, some patients develop dilated cardiomyopathy (DCM) after pacemaker implantation (PMI).[3,4,5] We also encountered four patients with isolated CCHB who developed DCM after PMI.

Our purpose in this retrospective study was to evaluate the clinical features of these patients, hoping to identify factors that might predict the development of DCM despite adequate PMI.

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Subjects and methods

Classification

In total, 47 patients were identified in National Cardiovascular Center, Osaka, Japan, as CCHB after PMI, and 15 patients with isolated CCHB were classified into two groups, one consisted of four patients who were diagnosed as having CCHB *in utero* or at birth and who developed DCM after adequate PMI (DCM group). The other group consisted of 11 patients with a similar clinical course as the DCM group but who did not develop DCM (non-DCM group).

None of the cases had anatomical cardiac abnormalities, including coronary anomalies. Pacemaker implantation was indicated due to arrhythmia (long QT syndrome, polymorphic premature ventricular contraction and/or atrial flutter) in four cases (two cases in DCM group), and low-output syndrome, congestive heart failure, syncope and/or ventricular volume overload due to severe bradycardia in the remaining 11 cases (two in the DCM group). With the exception of case 6,

PMI was performed with epicardial leads. Patients were diagnosed as having dilated cardiomyopathy clinically, based on symptoms of low-output syndrome and congestive heart failure, with cardiomegaly on chest X-ray and volume overload and low ejection fraction of the left ventricle at cardiac catheterization or on echocardiography. Finally, they had pathological findings compatible with DCM.

Clinical data accumulation

From the patient records, we obtained the following clinical data: gender, age at PMI, the presence or absence of maternal autoantibodies (anti-SSA antibody, anti-SSB antibody and antinuclear antibody) and the presence or absence of perfusion defects in 201-thallium or technetium-99 m myocardial imaging (MI). MI was performed in one patient in the DCM group twice at 0.4 and 59.9 months, and in four patients in the non-DCM group five times between 0.8 and 52.1 months before PMI. All four patients in the DCM group underwent MI five times between 0.8 and 43.9 months, and in four patients in the non-DCM group five times between 0.8 and 120.5 months after PMI. Ventricular and atrial heart rate was obtained from an electrocardiogram, which was done at 0–0.7 months (mean, 0.2 months) in the DCM group and 0–1.3 months (mean, 0.5 months) in the non-DCM group before PMI, and 3.7–48.8 months (mean, 22.9 months) and 19.1–270.8 months (mean, 115.6 months) after PMI. Pacing mode, pacing rate, width of QRS after initial PMI and the location of ventricular pacing were also recorded.

End-diastolic dimension (LVDd) and fractional shortening (%LVFS, %) of the left ventricle were obtained from an echocardiogram performed 0–2.4 months (mean, 0.6 months) in the DCM group and 0–2.8 months (mean, 0.9 months) in the non-DCM group before PMI, and 3.6–48.8 months (mean, 22.9 months) and 19.1–264.6 months (mean, 101.7 months) after PMI. The LVDd was expressed as a sex-matched percentage of the normal body surface area-predicted value (%LVDd, %).

We also reviewed the pathological findings at myocardial biopsy or autopsy. Endocardial or interstitial fibrosis was classified into four grades: no fibrosis (none), slight, moderate and severe.

We compared clinical data between the DCM group and the non-DCM group.

Data analysis

Comparisons between the two groups were performed using the Mann–Whitney *U*-test or Fisher's exact test. A *P*-value of < 0.05 was considered statistically significant.

Results (Tables 1, 2)

Dilated cardiomyopathy group

The patients were all male. The oldest patient (case 4) had a history of atrial flutter. PMI was done just after birth in two patients, at 2 months old in one patient, and at 7 years old in one patient. Maternal autoantibodies were present in two of the four patients. The ventricular rate before PMI was 44–53 bpm (mean, 47 bpm) and the atrial rate was 101–188 bpm (mean, 151 bpm). %LVDd was 116–143% (mean, 125%), and slight volume overload of the left ventricle was found in all. %LVFS was decreased to 18 and 21 in two patients, although severe congestive heart failure was not present in any patient. Single chamber pacing was employed in the three patients paced from infancy. Dual chamber pacing was used in one older patient. The initial pacing rate was 120 bpm in the three patients with single chamber pacing. DCM developed 2–43 months after PMI. A severe decrease in left ventricular contractility with dilatation was found (mean %LVFS, 8; mean %LVDd, 156%). Three patients died 7–48 months after PMI. The survivor is being treated with beta-blocker, angiotensin-converting enzyme inhibitor and diuretics. MI was done in one patient before PMI and in all patients after the development of DCM, and localized perfusion defects (PD) were found in all. In the one patient, there was no PD at 59.9 month before PMI, but a PD was detected 58 month later. There was a PD in all examinations in the DCM group except for one patient scanned at 0.8 month after PMI.

Biopsy or autopsy was done in all patients. All four patients showed slight to severe endocardial or interstitial fibrosis, and endocardial fibroelastosis was present in two patients. Trivial inflammatory cell infiltration was present in only one patient, but it was not thought to indicate myocarditis.

Non-dilated cardiomyopathy group

These 11 patients were all alive. In the patients who underwent PMI as a neonate or in early infancy, the indications for PMI were poor feeding and poor bodyweight gain due to bradycardia, and two patients (case 11, 14) had fetal distress and hydrops foetalis. Of the patients who received PMI when older (cases 6, 10, 12), the indications for PMI were volume overload of the left ventricle, and in one patient (case 6), a history of syncope. Autoantibodies were present in five and localized PD were found in all five patients in whom MI was done. Myocardial biopsy revealed interstitial fibrosis in one of two patients.

Comparison between groups

Comparing factors before PMI, no statistically significant differences were found in age at PMI or ventricular rate

Table 1 Patient profiles before pacemaker implantation

Patient	Gender	F/U period (mo)	Age at PMI (mo)	Auto-antibody	Before PMI				
					ECG		Echocardiography		MI
					HR (bpm)	P rate (bpm)	%LVDd (%)	%LVFS (%)	PD-location
DCM group									
1	m	37	2	(–)	45	188	121	32	ND
2	m	7	0	(–)	53	172	116	18	ND
3	m	48	0	(+)	49	143	143	21	ND
4	m	8	7 years 9 months	(+)	44	101	121	45	(+)
Mean ± SD		25 ± 20	23 ± 46		47 ± 4	151 ± 38	125 ± 12	29 ± 4	
non-DCM group									
5	f	274	2		40	149			ND
6	f	107	11 years 4 months		47	93	130	41	(+) – apical
7	f	199	0		53	135	99	30	ND
8	f	192	0		59	185			ND
9	m	164	1 year 7 months	(+) SSA	31	90	139	27	(+) – apical
10	f	75	2 years 9 months		50	115		35	(+) – anterolateral
11	f	102	0		55	115	155		ND
12	f	85	5 years 4 months	(+) SSA, ANA	38	85	124	44	(+) – apical
13	m	70	0	(+) SSA, ANA	52	136	121	32	ND
14	m	69	0	(+) SSA, ANA					ND
15	m	25	0	(+) SSA	53	109	124	44	ND
Mean ± SD		132 ± 76	23 ± 42		47 ± 8	121 ± 30	127 ± 16	36 ± 3	

ANA, antinuclear antibody; DCM, dilated cardiomyopathy; ECG, electrocardiography; f, female; F/U, follow-up; HR, heart rate; LVDd, end-diastolic dimension of left ventricle; LVFS, fractional shortening of left ventricle; m, male; MI, myocardial imaging; Mo, month; ND, not done; PD, perfusion defect; PMI, pacemaker implantation; SSA, anti-SSA antibody.

between the two groups. Atrial rate and %LVFS before PMI were not significantly different, but the atrial rate tended to be greater and %LVFS to be worse in the DCM group. Neither the initial mode of PM nor the width of QRS after PMI was significantly different between the two groups.

Discussion

CCHD is considered to have a good prognosis with appropriate management[1,6] but recently some cases that developed severe DCM despite early pacemaker implantation have been reported.[3,4] Some reports discuss risk factors for the development of DCM after adequate PMI, but the causes are unclear, making it important to evaluate the clinical features of these patients to identify factors that might predict the development of DCM despite adequate PMI.

In our study, 9% (4/47) of patients developed severe DCM after PMI, a frequency similar to previous reports.[3,6] Previous studies, concerned with DCM without CCHD, reported that approximately one-third of patients with DCM die.[7] In contrast, Udink *et al.* reported that 22% (2/9) of patients die, and Moak *et al.* also reported a 25% (4/16) mortality rate; and heart transplantation was done in 22% (2/9) and 44% (7/16), respectively, in their reports concerned with DCM with CCHD after PMI.[3,4] In our study, 75% (3/4) died, suggesting that the prognosis for patients with CCHD who develop DCM after PMI may be poorer than for DCM without CCHD.

Udink *et al.* reported that risk factors may include an early increased cardiothoracic ratio and left ventricle dilation, with little or no improvement in ventricular size with pacing, a prenatal diagnosis of CCHB, and bradycardia at birth.[3] Moak *et al.* examined possible serological, histological and electrophysiological risk factors but were unable to identify any significant predictors.[4] In our study, based on the MI and pathological findings, we suspect that the DCM patients with CCHB had pre-existing myocardial perfusion abnormalities or fibrosis of the myocardium before PMI. However, even in the non-DCM group, there were patients with suspected myocardial involvement. Thus, any relationship between myocardial involvement found in patients with CCHB and the development of DCM was unclear.

Table 2 Patient profiles after pacemaker implantation

Patient	Interval from PMI to P/O of DCM (Mo)	Prognosis	Interval from PMI to death (Mo)	Mode	Pacemaker		Width of QRS (ms)	After PMI			Biopsy/Autopsy	
					Lead position	HR (bpm)		Echocardiogram %LVDd (%)	%LVFS (%)	MI PD	Fibrosis	Hypertrophy
DCM group												
1	30	Death	37	VVI	RV		112	149	7	(+)	sl-moderate (EFE)	(-)
2	6	Death	7	VVI		120		176	5	(+)	severe (EFE)	(-)
3	43	Death	48	VVI	RV	120	80	155	6	(+)	severe	(-)
4	2	Alive		DDDR	RV		120	142	15	(+)	slight	(+)
Mean ± SD	20 ± 19		30 ± 21		120	104 ± 21	156 ± 14	8 ± 4				
non-DCM group												
5		Alive		VVI		100	120	107	32	ND	ND	ND
6		Alive		DDD	RV		120	107	31	ND	(+)	ND
7		Alive		VVI	RV	110	80	102	43	(+)	ND	ND
8		Alive		VVI	RV	110	100	90	25	ND	ND	ND
9		Alive		DDD			120	96	35	(+/-)	ND	(+)
10		Alive		VVI	LV	90	80	118	30	(+)	ND	ND
11		Alive		VVI	RV	110	112	86	32	ND	ND	ND
12		Alive		DDD	LV		112	105	52	ND	ND	ND
13		Alive		VVI		110	88	104	36	ND	ND	ND
14		Alive		VVI	RV	120	88	92	46	ND	ND	ND
15		Alive		VVI	RV	120	104	95	40	ND	ND	ND
Mean ± SD						109 ± 10	102 ± 15	100 ± 9	36 ± 2			

DCM, dilated cardiomyopathy; EFE, endocardial fibroelastosis; HR, heart rate; LV, left ventricle; LVDd, end-diastolic dimension; LVFS, fractional shortening of left ventricle; MI, myocardial imaging; Mo, month; ND, not done; PD, perfusion defect; PMI, pacemaker implantation; P/O, point out; RV, right ventricle.

Taylor *et al.* reported an association between autoantibodies and the development of DCM in patients with CCHB after PMI.[8] We found maternal autoantibodies in all cases that were examined in the non-DCM group while autoantibodies were not present in two cases in the DCM group. While the presence of maternal autoantibodies may be a risk factor for myocardial involvement in patients with CCHB, they are not the cause of the development of DCM. The titer of autoantibodies or the timing and period of exposure to autoantibodies *in utero* may determine the extent of myocardial damage and/or the development of DCM, though this possibility could not be examined in our study.

Clinical factors prior to PMI (age at PMI, cardiac size, cardiac function and the exact rhythm) were not associated with the development of DCM after PMI statistically, although the atrial rate and LVFS data might suggest that the DCM group was actually more ill before PMI subclinically.

Recently, the clinical benefits of cardiac resynchronization therapy for heart failure were reported.[9] In this regard, atrioventricular and/or inter/intraventricular desynchronization may be considered a cause of heart failure, and atrioventricular and inter/intraventricular resynchronization with dual chamber and/or biventricular pacing may produce hemodynamic benefits. Manolis *et al.* suggested an association between the mode and site of pacing and coronary flow.[10] In this respect, ventricular single chamber pacing is less desirable than atrial or sequential dual chamber pacing because it may be associated with abnormal conduction and pacing, and may itself be a factor in causing DCM after PMI. Of our patients, pacing was converted from single chamber pacing to dual in one (case 2), but congestive heart failure was so severe before conversion that there was no improvement. Sequential dual chamber pacing or biventricular pacing may be more effective in preventing the development of DCM after PMI. Further experience will determine whether conversion of pacing mode improves the course of congestive heart failure. To evaluate any association between pacing itself and the development of DCM after PMI, further studies in patients with acquired CHB are necessary.

Study limitation

Our study involves only a few patients in each group. Studies involving larger patient numbers may uncover factors that predict the risk of DCM after adequate PMI.

Summary

We report four CCHB cases that developed DCM after PMI. We suspect that the patients with CCHB had pre-existing myocardial perfusion abnormalities or myocardial fibrosis,

although no apparent risk factor predicting the development of DCM after PMI was identified. The presence of maternal autoantibodies may correlate with myocardial involvement. Pacing itself may be implicated in the development of DCM after PMI in patients with CCHB. Patients with CCHB require careful follow up for both arrhythmia and cardiac function after PMI, even after early and adequate PM initiation.

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治療の歴史

血管新生療法

竹下　聡

はじめに

血管新生療法（therapeutic angiogenesis）[1]は，血管増殖因子やその遺伝子，あるいは骨髄や末梢血細胞を用いて血管新生を促進させ，組織虚血の改善を図る治療法である。循環器領域における初の遺伝子治療としても知られるvascular endothelial growth factor（VEGF）遺伝子を用いた血管新生療法が，米国のIsnerらによって行われたのは1994年のことである[2]。現在までにすでに10年以上が経過し，遺伝子以外にも増殖因子蛋白，骨髄細胞，末梢血細胞などを用いたさまざまな治療が試みられ，その有効性も検証されつつある。各々の治療法の詳細は他稿に譲り，ここでは血管新生療法がどのように生まれ，どのように育ってきたか，その歴史を概説する。

血管新生療法の臨床応用まで

血管新生療法のコンセプトそのものは決して新しいものではない。80年代後半には，ネコの虚血肢モデルに対して大網の脂質分画を投与し，虚血を改善させる試みが行われている。大網や脂肪細胞の再生医療への応用は最近のトピックであり，このような研究がすでに20年近く前に存在したことは興味に値する。これらの血管新生療法とIsnerらが行ったそれとの差異は，後者がVEGFという血管内皮細胞に特異的な増殖因子を用いた点にある。90年代初頭，Isnerらは家兎の虚血肢モデルにVEGF蛋白を投与し，血管新生療法の臨床応用を検討した。動脈投与，静脈投与，繰り返し投与，ヘパリンの併用などさまざまな投与法を検討し，投与法のいかんに関わらず，側副血行の促進には100～1000 μgのVEGF蛋白が必要なことを明らかにした。しかしながら，大量のVEGF蛋白を投与すると，投与した蛋白が全身を循環し，非目的部位へと到達するのは避け難い。血管増殖因子の全身への拡散は，糖尿病患者においては網膜症を悪化させ，癌患者では腫瘍血管の発達を促進させる。また，一部の血管増殖因子はNOを介した血管拡張作用を有し，遷延性低血圧を惹起する。事実，VEGF蛋白を用いた血管新生療法の臨床試験では，低血圧を避けるために投与量が制限された。

大量の蛋白投与に伴う副作用を回避するために行き着いた結論が遺伝子を用いたローカルドラッグデリバリーであった。Isnerらはカテーテルを用いて血管細胞へVEGF遺伝子を経皮的に導入し，それらの細胞からVEGF蛋白を分泌させようと考え，表面が親水性ゲルでコーティングされた冠動脈形成術用バルーンカテーテル（ハイドロゲル・バルーンカテーテル）による遺伝子導入を試みた。ハイドロゲルは，狭窄部位におけるバルーン通過性を改善するために施されたコーティングであるが，Isnerらはこのゲルにプラスミドの水溶液をしみ込ませ，遺伝子キャリアとして用いた。通常のPTCAテクニックによりバルーンを目的部位へと進め，4～8気圧で1分間バルーンを拡張させることで遺伝子の血管壁への導入が可能であった。その導入効率はリポゾームによる遺伝子導入に比し100倍以上の高効率ではあったが，βガラクトシダーゼ遺伝子を用いた組織的検討では，導入部位のわずか0.1%以下の細胞にしか遺伝子発現が認められなかった[3,4]。このわずかな細胞によって血管新生を促進することが可能なのかが問題となるのだが，遺伝子の導入効率（transfection efficiency）と治療効率（therapeutic efficiency）とは同義ではない。遺伝子産物である増殖因子が細胞外へと分泌されれば，たとえ導入効率は低くとも，パラクリン効果が期待できる[5]。この仮説は動物実験によって検証された。すなわち，ハイドロゲル・バルーンカテーテルを用いて家兎虚血肢モデルにVEGF遺伝子の導入を行うと，約3週間にわたりその発現が認められ，VEGF蛋白の動脈内投与と同等以上の側副路発達効果が得られたのである。一方，末梢血中のVEGF蛋白の濃度はELISAによる測定限界付近にあり，きわめて低値であった。つまり遺伝子の導入効率は低くとも，治療効果を得るに十分なVEGFの局所濃度が維持可能であり，逆に末梢血中濃度は希釈効果によって低く抑えられたのであ

たけした さとし：国立循環器病センター心臓血管内科

る。ここで忘れてならないのは，本法がプラスミドDNA以外には何らベクターを用いない遺伝子導入法（naked DNAアプローチ）である点で，臨床応用における高い安全性が期待された。

末梢動脈閉塞症に対するVEGFを用いた血管新生療法

1994年，Isnerらは血管新生療法の臨床試験を開始した[2]。この臨床試験は，循環器領域における初の遺伝子治療としても知られており，内科治療や外科治療不応性の重症末梢動脈閉塞症患者を対象として行われた。遺伝子治療から1〜2ヵ月で，血管造影上の新生血管出現が得られ，下肢疼痛や難治性潰瘍が消失した。副作用は下腿浮腫や良性血管腫など，一過性の軽微なものであった。しかしながら，バルーンカテーテルを用いた遺伝子導入は，動脈穿刺が不可能な例，下肢の動脈硬化が高度でカテーテルによるアプローチが困難な例，遺伝子導入に際し解離などの血管損傷リスクが高い例などには施行できない。そこで考案されたのが，虚血筋への遺伝子導入である[6]。Baumgartnerらは，VEGFプラスミドの筋注により，7〜8割の症例で血管造影上の側副路発達や臨床症状の改善を得ることに成功した[7]。この遺伝子導入法の単純化により，カテーテルでは治療困難であった症例にも血管新生療法が可能となり，その適応は大きく拡大することとなる。また，本法は心筋へも応用可能であり，虚血性心疾患に対する血管新生療法の臨床応用への契機となった。

虚血性心疾患に対するVEGFを用いた血管新生療法

90年代後半，Losordoらは胸部小切開法により重症狭心症患者の左室にVEGFプラスミドを筋注し，狭心症状の著明な改善と心筋シンチによる虚血所見の改善を得ることに成功した[8]。さらにLosordoらは，NOGAと呼ばれる心筋マッピングのシステムを用いて，心内膜側から経皮的にVEGF遺伝子の導入を行い，良好な治療効果を得た[9]。現在，このNOGAシステムを用いた経皮的遺伝子治療は，二重盲検試験によって検証中である。最近，類似のプロトコールを用いた臨床試験の結果がKastrupらにより報告されたが，VEGF治療群において左室壁運動の改善は認められたものの，自覚症状や心筋シンチ所見の改善は得られていない[10]。本法の有効性については，さらなる検討が必要である。

血管内皮前駆細胞の発見と細胞治療

遺伝子を用いた血管新生療法の臨床応用が進むなか，1997年，Asaharaらはヒト末梢血中のCD34陽性細胞の分画中に成熟内皮細胞へと分化しうる血管内皮前駆細胞（endothelial progenitor cell：EPC）が存在することを明らかにした[11]。これを契機として，遺伝子や蛋白を中心とした血管新生療法に，細胞移植を用いた血管新生療法の新しい流れが加わった。

EPCは血球血管芽細胞（ヘマンジオブラスト）と呼ばれる幹細胞より分化するが，成人では通常骨髄中にあり，末梢血中にはきわめてわずかしか存在しない。Kalkaらはヒト末梢血単核球からEPCを分離培養し，マウスの虚血肢モデルに投与することで下肢虚血の改善を得た[12]。一方，Shintaniらは自己骨髄由来単核球移植によって家兎虚血肢の血管新生が増強することを報告した[13]。移植された自己骨髄単核球が虚血組織における血管形成に参加，もしくは血管増殖因子を放出することで局所の血管新生を刺激したものと思われ，自己骨髄単核球細胞移植による血管新生療法の臨床応用への契機となった。

自己骨髄単核球細胞移植による血管新生療法

末梢動脈閉塞症に対する自己骨髄細胞移植の有用性は，2000年，国内3施設（久留米大学，関西医科大学，自治医科大学）によるTherapeutic Angiogenesis Using Cell Transplantation (TACT) trialにおいて示された[14]。全身麻酔下で採取した数百ccの骨髄液から単核球を分離後，虚血肢に移植することで，ABI（上肢・下肢血流比）は0.97ポイント増え，トレッドミル歩行距離は2.6倍に改善した。また，下肢疼痛は9割，皮膚潰瘍は8割の症例で改善した。同様のプロトコールを用いた多施設臨床試験がすでに実施されており，少なくとも本法の短期成績に関しては確立された治療法といっても過言ではない。

末梢血細胞を用いた血管新生療法の臨床応用に関しては，顆粒球コロニー刺激因子（granulocyte colony stimulating factor：G-CSF）を用いて末梢血中の単核球からCD34陽性細胞を分離したり，末梢血単核球細胞移植にアドレノメデュリンの局所投与を併用するなどさまざまな試みがなされている。その有効性に関してはまだ不明な点が多いものの，侵襲性の低さや細胞採取の容易さなど末梢血細胞移植の

メリットは大きく，今後の発展が期待される。

一方，虚血性心疾患に対する細胞治療に関しても，骨髄細胞の冠動脈内注入やNOGAシステムを用いた心筋内移植など，さまざまな臨床試験が進行中である。急性心筋梗塞患者の冠動脈内に骨髄単核球細胞を投与した初期の臨床試験では，梗塞サイズの減少や左室機能の改善，心筋バイアビリティーの改善が報告されているが，その治療効果については否定的な報告も少なくない。また，左室機能改善などの治療効果が血管新生によって得られたものなのか，あるいは心筋細胞の再生によるものなのか，その機序についても不明な点は多い。虚血下肢に対する細胞移植ほど確立された治療にはまだ至っていないというのが現状である。

おわりに

血管新生療法は血管増殖因子を用いた遺伝子治療として幕を開けた。しかしながら，遺伝子のパテント問題や倫理的ハードルの高さから，現在では細胞移植による血管新生療法が主流となりつつある。

虚血下肢に対する細胞移植の治療成績は良好であるが，臨床症状の改善にもかかわらず血管造影での改善を認めないことも少なくない。果たして細胞移植により血管新生が本当に促進されたのか？　単に潰瘍の創傷治癒機転が促進されただけではないのか？　その治療機序に関してはいまだ不明な点が少なくはなく，今後の研究成果が期待される。

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心血管疾患における細胞–遺伝子ハイブリッド治療

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はじめに

1980年代より遺伝性疾患を皮切りに，悪性腫瘍，自己免疫疾患などに対して遺伝子治療の臨床応用が開始され，現在では欠損遺伝子や変異遺伝子を補充する治療法から，生体の治癒力を補う目的の治療にも拡大施行されている．循環器疾患における遺伝子治療は，1994年に米国のタフツ大学でVEGF（血管内皮細胞増殖因子）遺伝子の虚血肢への血管内投与という形で行われ，血管再生効果が確認された[1]．以後，循環障害に対する遺伝子治療の臨床応用が多くの施設で行われている．

また，日本では1997年に臓器移植法が施行され，心臓，肺などの脳死臓器移植が可能となったが，ドナー不足のためにこれまで二十数例が施行されるにとどまっている．末期の臓器不全に対して何らかの代替治療が必要とされている中で，体性幹細胞移植やtissue-engineeringなどの再生医療が注目を集めている．当施設では，2004年より既存の治療に抵抗性の重症拡張型心筋症および虚血性心筋症の患者に対して，間葉系幹細胞移植の臨床研究を開始している．

われわれはこの遺伝子治療と細胞移植治療をハイブリッドし，基地（base）となる機能細胞に補完的機能をもつ遺伝子を導入するという遺伝子–細胞ハイブリッド治療を考案した．遺伝子を格子構造を有する生分解性ゼラチンに取り込ませ，このゼラチン–遺伝子複合体を貪食能を有する細胞に導入させる方法である．われわれは正常な血管床の再生という点に主眼を置き，細胞–遺伝子ハイブリッド治療を原発生肺高血圧症に応用した．

本稿では，ゼラチンを用いた遺伝子導入法，ゼラチン–遺伝子複合体，肺高血圧症に対する細胞–遺伝子ハイブリット治療の研究成果について概説する．

ゼラチンを用いた遺伝子導入法

従来用いられてきた代表的な遺伝子導入法として，プラスミドDNAそのものを組織に投与する方法，アデノウイルスやレトロウイルスなどのウイルスベクターを用いる方法などがあげられる．しかし，プラスミドDNAの直接投与では組織内で拡散・希釈され，細胞核内に到達する前に核酸分解酵素により分解されてしまうため，有効な治療効果を得るには大量の遺伝子を準備する必要がある．また，ウイルスベクター法は導入効率は良いものの，ウイルス蛋白の抗原性，ウイルスの突然変異の懸念など，安全性に重大な問題がある．われわれが考案した細胞–遺伝子ハイブリッド化は，*ex vivo*でウイルスベクターを用いることなく細胞への遺伝子導入が高効率に行える次世代の

[*Key words*]　細胞–遺伝子ハイブリッド治療，血管新生，肺高血圧症

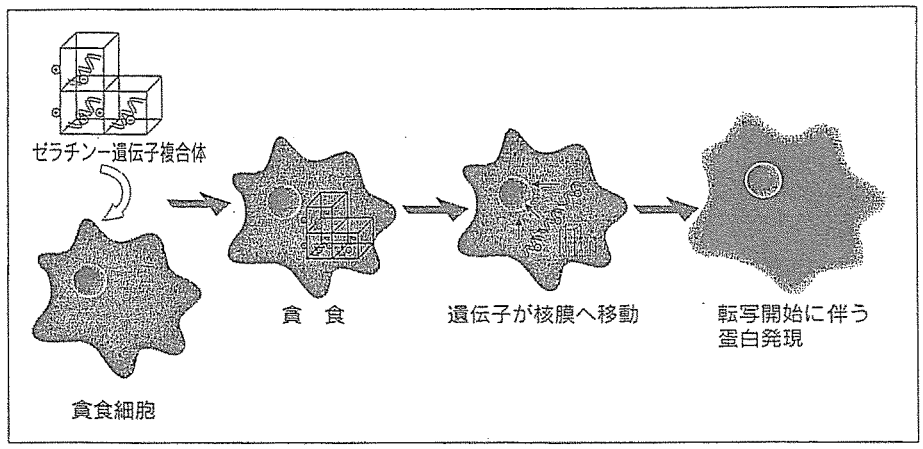

図1　ゼラチンを用いた遺伝子導入の概念図
貪食細胞がゼラチン−遺伝子複合体を取り込んだ後，ゼラチンは細胞内で分解され，封入されていた遺伝子が核膜へ向かい，蛋白発現に向けたプロセスが開始される．

遺伝子導入法である（図1）．このハイブリッド化を実現する鍵となる物質が生分解性ゼラチンである．

ブタの皮膚から抽出したゼラチンをグルタルアルデヒドの架橋反応により格子構造とし，エチレンジアミンを加えると正帯電ゼラチンが完成する[2]．この正帯電ゼラチンの特徴として，

① 陽性に帯電しているので，陰性に帯電しているDNAや蛋白質と数時間接触することにより容易にイオン結合し，電気的複合体を形成する．
② 構造が3次元格子状なので，結合物質をゲル内部に保護することにより，分解酵素の影響を受けにくくする．
③ 生体内で徐々に分解を受けて，この分解に伴い結合物質を徐々に放出する．
④ その分解速度はゼラチンの架橋度を変えることにより自由に調節できる．
⑤ ゼラチン−遺伝子複合体は貪食細胞（単球，マクロファージなど）に容易に貪食される．
⑥ 貪食細胞内で高率に遺伝子を発現する．

などがあげられる．

われわれはゼラチンを，その構造や表面電荷を自由に変えることが容易である性質から遺伝子の担体として利用することを着想した．遺伝子をあらかじめゼラチンの格子構造内へ封入してゼラチン−遺伝子複合体を形成し，生体内へ投与することで核酸分解酵素による遺伝子の分解・代謝が緩徐となり，結果として安全かつ高効率に遺伝子を導入することができると考えられる．実際に遺伝子をゼラチンと結合させて投与したところ，生体内における遺伝子の残存期間を延長させることに成功し，遺伝子の発現率も従来の遺伝子単独投与と比較して約10倍の増加が認められた[3]．

ゼラチン−アドレノメデュリン遺伝子複合体による血管新生療法

血管内皮細胞から産生されるアドレノメデュリンは，生体内で最も強い血管拡張作用を示すペプチドであり[4]，血管新生促進や抗アポトーシス作用などの多様な生理活性を併せもつ．われわれは家兎下肢虚血モデルを用いてゼラチン−アドレノメデュリン遺伝子複合体の治療効果を検討した[5]．大腿動脈摘除後10日目に大腿筋肉内にアドレノメデュリン遺伝子そのもの，およびゼラチン−アドレノメデュリン遺伝子複合体を投与した群を作製し，4週間後に下腿血圧，組織血流量および組

織内の毛細血管密度を調べた．いずれの治療もコントロール群と比較して著明な血管新生および血流改善効果を認めたが，ゼラチン-アドレノメデュリン遺伝子複合体投与群が有意に勝っており（図2a, b, c），血管造影でもより多くの再生血管が描出された（図2d）．また，筋肉組織中のアドレノメデュリン濃度は投与後2週間にわたって遺伝子単独投与群よりも高値を示した（図2e）．この事実により，アドレノメデュリン遺伝子をゼラチン内に封入して投与することで，遺伝子が虚血組織内で長期にわたり高濃度に維持され，徐放されながら遺伝子導入を果たし，より効果的な血管新生の発現が得られたと考えられる．

ゼラチンを介した細胞-遺伝子ハイブリッド治療

遺伝子または調節因子の投与のみでは代謝による影響があり，必要とされる場所への有効量の到達の面で限界がある．この問題を解決するために1991年に細胞を遺伝子発現の基地として用いるcell-basedの遺伝子治療がPlautzらによって始められた[6]．これをさらに発展させた細胞-遺伝子ハイブリッド治療は，貪食能をもつ機能細胞にゼラチン-遺伝子複合体を取り込ませて遺伝子導入を行う．つまり，細胞が遺伝子発現の基地としてだけではなく，治療要素としての働きをももつことになる．細胞移植の観点から言い換えれば，移植細胞の機能強化のために遺伝子治療を併用した治療法といえる．細胞-遺伝子ハイブリッド治療は，ベクターにウイルスを使用せず，ゼラチンを用いることで安全性と効率の高い遺伝子導入を実現する．また，移植したマクロファージや単球などの貪食細胞が自身の走化性によって障害部位に特異的に集まるため，標的組織へ凝集したこれらの細胞が導入された遺伝子をもとにタンパク質を合成し，より高い治療効果を上げると考えられる．

血管内皮前駆細胞を用いた原発性肺高血圧に対する細胞-遺伝子ハイブリッド治療

1997年Asaharaらは，血管内皮前駆細胞（endothlial progenitor cells: EPCs）が生体内で虚血や血管内皮障害が起こったときに骨髄から末梢血中に動員され，障害部位に遊走・付着し，血管内皮細胞に分化して血管を形成することを明らかにした[7,8]．また，われわれはEPCsがマクロファージのような貪食能を有し，ゼラチン-遺伝子複合体を貪食することを発見した[9]．EPCsの移植は虚血性心疾患，閉塞性動脈硬化症の治療に有効であることが報告されており[10,11]，この効果は，①EPCs自身が血管形成に加わること，②EPCsがVEGFなどの血管新生因子を放出して局所の血管新生を促すためと考えられる．

原発性肺高血圧症の病態は，血管内皮細胞の機能障害およびそれに基づく血管作動物質の不均衡であると考えられている（図3）．われわれは，肺血管床で強力な拡張因子として働くアドレノメデュリンを血管内投与することで，平均肺動脈圧を低下させることができることを示してきた[12]．アドレノメデュリンの特異的受容体は，体血管よりもむしろ肺血管に多数存在し[13]，血管平滑筋の受容体に直接作用してcAMPを増加させたり，血管内皮細胞に働き一酸化窒素を介して血管拡張を引き起こす[14]．故に，原発性肺高血圧症は細胞-遺伝子ハイブリッド治療が適している疾患と考えられた．

まず，アドレノメデュリン遺伝子をゼラチンに封入して*ex vivo*にてEPCsに取り込ませた（図4a, b, c）．このEPCsの貪食による遺伝子導入は，ウイルスベクターを用いずにEPCs自身への50～70%という高効率の遺伝子導入を可能にした．近年アドレノメデュリンはPI3K-Akt経路を活性化することで血管内皮細胞の生存，遊走，増殖に関与することがわかり，アドレノメデュリン遺伝子を導入することでEPCs自身のアポトーシス抑制，増殖促進の効果も得られることが明ら