Results

Patients Characteristics

Table 1 shows the baseline characteristics of the two groups. There were no significant differences in age, blood pressure, serum creatinine, and hemoglobin between 10-mg and 20-mg group of cilnidipine. None of the patients had the clinical side effects of cilnidipine.

Effects of Cilnidipine on Blood Pressure and Heart Rate

After the treatment with cilnidipine for 6 months, blood pressure was significantly reduced in all patients, and the effect of blood pressure lowering was significantly greater in 20-mg group than in 10-mg group (Tables 2, 3, and 4). Heart rate was not significantly decreased in both groups after the treatment with cilnidipine (Tables 2, 3, 4).

Effects of Cilnidipine on Sympathetic and Parasympathetic Nerve Activity

After the treatment with cilnidipine for 6 months, LFnuSBP were significantly decreased in both groups (Tables 2 and 3), and the suppressive effects were stronger in the 20-mg group than in the 10-mg group (Table 4). While HFnuRRI was not significantly changed in the 10-mg group (Table 2), it was significantly increased in the 20-mg group (Table 3).

Effects of Cilnidipine on Baroreflex Sensitivity

In the 10-mg group, BRS was not significantly changed between before and after the treatment with cilnidipine (Table 2). However, in the 20-mg group, BRS was significantly improved after the treatment with cilnidipine for 6 months (Table 3).

Table 1
Clinical profile of the patients in 10-mg and 20-mg group

	10-mg Group (n = 5)	20-mg Group (n = 5)	
Age (year)	56 ± 7	59 ± 8	NS
Systolic blood pressure (mmHg)	161 ± 13	157 ±12	NS
Diastolic blood pressure (mmHg)	100 ± 5	98 ± 8	NS
Heart rate (bpm)	80 ± 5	76 ± 6	NS
AST/ALT (IU/L)	26 ± 11/27 ± 13	26 ± 9/28 ± 6	NS
Cr (mg/dL)	0.7 ± 0.2	0.8 ± 0.2	NS
Total cholesterol (mg/dL)	182 ±19	178 ± 22	NS
Triglyceride (mg/dL)	92 ± 33	88 ± 36	NS
LDL cholesterol (mg/dL)	110 ± 14	108 ± 12	NS
HDL cholesterol (mg/dL)	46 ± 7	48 ± 6	NS
Glucose (mg/dL)	89 ± 11	93 ± 10	NS
HbA1c (%)	5.6 ± 0.4	5.5 ± 0.6	NS
BNP (pg/ml)	38 ± 14	32 ± 11	NS
Left ventricular ejection fraction (%)	70 ± 9	72 ± 8	NS
Cardio-thoracic ratio(%)	54 ± 8	51 ± 8	NS

Table 2
Changes in blood pressure, heart rate, and autonomic function in the patients with 10-mg group

	Pretreatment (n = 5)	Cilnidipine 10 mg (n = 5)	P
Systolic blood pressure (mmHg)	161 ± 13	137 ± 13	< 0.05
Diastolic blood pressure (mmHg)	100 ± 5	87 ± 4	< 0.05
Heart rate (bpm)	80 ± 5	76 ± 7	NS
HF-RR (ms2)	102 ± 63	106 ± 58	NS
HFnuRR (%)	39 ± 6	42 ± 6	NS
LF-SBP (mmHg2)	0.7 ± 0.3	0.5 ± 0.5	NS
LFnuSBP (%)	56 ± 5	49 ± 4	< 0.05
Baroreflex sensitivity (ms/mmHg)	14.2 ± 2.6	16.2 ± 4.8	NS

Table 3
Changes in blood pressure, heart rate, and autonomic function in the patients with 20-mg group

	Pretreatment (n = 5)	Cilnidipine 20 mg (n = 5)	P
Systolic blood pressure (mmHg)	157 ± 12	120 ± 13	< 0.05
Diastolic blood pressure (mmHg)	98 ± 8	81 ± 5	< 0.05
Heart rate (bpm)	76 ± 6	73 ± 6	NS
HF-RR (ms2)	92 ± 44	102 ± 66	NS
HFnuRR (%)	39 ± 3	44 ± 2	< 0.05
LF-SBP (mmHg2)	0.6 ± 0.4	0.4 ± 0.3	NS
LFnuSBP (%)	63 ± 6	50 ± 4	< 0.05
Baroreflex sensitivity (ms/mmHg)	13.6 ± 2.9	20.2 ± 2.1	< 0.05

Table 4
Degree of changes in blood pressure, heart rate and autonomic function in the patients with 10-mg and 20-mg group

	Cilnidipine 10 mg (n = 5)	Cilnidipine 20 mg (n = 5)	P
Systolic blood pressure	−15%	−24%	< 0.05
Diastolic blood pressure	−13%	−17%	< 0.05
Heart rate	−5%	−4%	NS
HFnuRR	+7%	+13%	< 0.05
LFnuSBP	−12%	−12%	< 0.05
Baroreflex sensitivity	+14%	+49%	< 0.05

Discussion

In the present study conducted among patients with essential hypertension, cilnidipine produced a significant reduction in blood pressure with the inhibition of sympathetic nerve activity and the improvement of impaired baroreflex control. This study was the first to report that cilnidipine treatment achieved the inhibition of sympathetic nerve activity and the improvement of the impaired baroreflex control in the patients with hypertension. These results suggest that cilnidipine is preferable for the treatment with hypertension among the Ca channel blockers.

Epidemiological studies have demonstrated that a higher heart rate is associated with a long-term risk of cardiovascular mortality, independent of other cardiac risk factors (22). Therefore, anti-hypertensive drugs that do not increase the heart rate would seem to be preferable. It has been reported that the treatment with short-acting Ca channel blockers may not prevent cardiovascular disease (23,24). Accordingly, long-lasting Ca channel blockers that exert less influence on the sympathetic nervous system are now recommended for the treatment of hypertension. Amlodipine and cilnidipine, which were known as long-acting Ca channel blockers, were reported not to increase heart rate. Eguchi et al. (27) reported that cilnidipine did not cause reflex tachycardia, and that cilnidipine, but not amlodipine, significantly decreased the ambulatory BP level without causing an increase in heart rate. In this study, cilnidipine did not increase heart rate, and caused a significant decrease in the LFnuSBP, as the marker of the sympathetic nerve activity. Our results of the sympatho-inhibitory effects of cilnidipine were similar to the previous reports which calculated the sympathetic nerve activity by other methods. From these results, cilnidipine is considered to be the preferable drug with the sympatho-inhibtory effect among the Ca channel blockers.

In this study, BRS was improved in the patients with hypertension treated with high-dose cilnidipine. A previous study reported that BRS values calculated by sequence analysis had reasonable reproducibility when up and down sequences were combined (25), and we measured the BRS by sequence analysis. It has been reported that BRS is impaired in the patients with hypertension (17,26–29), and that BRS is the predictive factor of mortality and cardiovascular events (17). The results of this study suggest that cilnidipine is preferable for the treatment of hypertension among the Ca channel blockers. Previous studies suggested BRS measured by the sequence method was impaired in the patients with hypertension (5–12 ms/mmHg) (27–29), and BRS obtained in this study was considered to be higher compared to that in those previous studies. This difference may be due to the patients' characteristics in this study. The patients in this study had no complications and their hypertension was in early stages.

The mechanisms in which cilnidipine inhibits the sympathetic nerve activity may be due to suppressing the release of catecholamines from sympathetic nerve endings by blocking the N-type calcium channels distributed widely in sympathetic nerves (30). Recent studies have demonstrated the beneficial effect of cilnidipine on cardiac sympathetic nerve activity and cardiovascular morbidity (31–33). Sakata, Yoshida, and Obayashi reported that cilnidipine suppressed cardiac sympathetic overactivity while amlodipine had little suppressive effect (32). The effect of cilnidipine on heart rate might be due to not only long-acting effects but also to a reduction in sympathetic nerve activity. The mechanisms in which cilnidipine improved the BRS has not been determined in this study. Our previous study in animal models indicated that sympatho-inhibition causes the improvement of BRS in hypertensive model rats (26). Further clinical studies are necessary.

There are several limitations in this study. First, this study was a small-size, nonrandomized study. To establish the sympatho-inhibitory effect and improvement of BRS of cilnidipine, a randomized study is required. Second, the ages of the patients in this study were relatively young, and none of them had organ damage due to hypertension. Whether the treatment with cilnidipine causes the beneficial effects in this study in the older and complicated patients with hypertension has not been determined. Third, we determined the effects of cilnidipine on autonomic function for only 6 months, and only at two points, before and after 6 months. The blood pressure-lowering effect of cilnidipine is determined in several days after the initiation of administration (3). In the present study, we examined the effects of cilnidipine on the autonomic function at only two points pre—administration and after 6 months. From the results of the present study, we have not determined whether the mechanisms of the action of cilnidipine is similar between several days and 6 months after the initiation of cilnidipine. Furthermore, the effects of cilnidipine on the autonomic function for longer periods must be determined.

Conclusion

The treatment with cilnidipine for essential hypertension produced a significant reduction in blood pressure with the inhibition of sympathetic nerve activity and the improvement of impaired baroreflex control. These results suggest that cilnidipine is preferable for the treatment of hypertension among the Ca channel blockers.

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Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1. Staessen JA, Wang JG, Thijs L. Cardiovascular prevention and blood pressure reduction: a quantitative overview updated until March 2003. *J Hypertens.* 2003;21:1055–1076.
2. Yoshimoto R, Hashiguchi Y, Dohmoto H, Hosono M, Iida H, Fujiyoshi T, Ikeda K, Hayashi Y. Effects of a new dihydropyridine derivative, FRC-8653, on blood pressure in conscious spontaneously hypertensive rats. *J Pharmacobio Dyn* 1992;15:25–32.
3. Tominaga M, Phya Y, Tsukashima A, Kobayashi K, Takata Y, Koga T, Yamashita Y, Fujishima Y, Fujishima M. Ambulatory blood pressure monitoring in patients with essential hypertension treated with a new calcium antagonist, cilnidipine. *Cardiovasc Drugs Ther* 1997;11:43–48.
4. Hosono M, Fujii S, Hiruma T, Watanabe K, Hayashi Y, Ohnishi H, Takata Y, Kato H. Inhibitory effect of cilnidipine on vascular sympathetic neurotransmission and subsequent vasoconstriction in spontaneously hypertensive rats. *Jpn J Pharmacol* 1995;69:127–134.

5. Oike M, Inoue Y, Kitamura K, Kuriyama H. Dual action of FRC8653, a novel dihydropyridine derivative, on the Ba2+ current recorded from the rabbit artery. *Circ Res* 1990;57:993–1006.
6. Fujii S, Kameyama K, Hosono M, Hayashi Y, Kitamura K. Effect of cilnidipine, a new dihidropyrydine Ca++ channel antagonist, on N-type Ca++ channel in rat dorsal root ganglion neurons. *J Pharmacol Exp Ther* 1997;280:1184–1191.
7. Uneyama H, Takahara A, Dohmoto H, Yoshimoto R, Inoue K, Akaike N. Blockade of N-type Ca++ current by cilnidipine (FRC-8653) in acutely dissociated rat sympathetic neurons. *Br J Pharmacol* 1997;122:37–42.
8. Mancia G, Parati G, Castiglioni P, Di Rienzo M. Effect of sinoaortic deervation on frequency-domain estimates of baroreflex sensitivity in conscious cats. *Am J Physiol* 1999;276:H1987–H1993.
9. Laude D, Elghozi JL, Girard A, Bellard E, Bouhaddi M, Castiglioni P, Cerutti C, Cividjian A, Di Rienzo M, Fortrat JO, Janssen B, Karemaker JM, Leftheriotis G, Parati G, Persson PB, Porta A, Quintin L, Regnard J, Rudiger H, Stauss HM. Comparison of various techniques used to estimate spontaneous baroreflex sensitivity. *Am J Physiol* 2004;286:R226–R231.
10. Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. Heart rate variability: standards of measurement, physiological interpretation, and clinical ise. *Eur Heart J* 1996;17:354–381.
11. Lucini D, Meta GS, Malliani A, Pagani M. Impairment in cardiac autonomic regulation preceding arterial hypertension in humans: insights from spectral analysis of beat-by-beat cardiovascular variability. *Circulation* 2002;106:2673–2679.
12. Radaelli A, Perlangeli S, Cerutti MC, Mircoli L, Mori I, Boselli K, Bonaita M, Terzoli L, Candotti G, SIgnorini G, Ferrari AU. Altered blood pressure variability in patients with congestive heart failure. *J Hypertens* 1999;17:1905–1910.
13. Castiglioni P, Di Rienzo M, Veicsteinas A, Parati G, Merati G. Mechanisms of blood pressure and heart rate variability: an insight from low-level paraplegia. *Am J Physiol* 2007;292: R1502–R1509.
14. Latinen T, Hartikainen J, Vanninen E, Niskanen L, Geelen G, Lansimies E. Age and gender dependency of baroreflex sensitivity in healthy subjects. *J Appl Physiol* 1998;84:576–583.
15. Mortara A, La Rovere MT, Pinna GD, Prpa A, Maestri R, Fedo O, Pozzoli M, Opasich C, Tavazzi L. Arterial baroreflex modulation of heart rate in chronic heart failure: clinical and hemodynamic correlates and prognostic implications. *Circulation* 1997;96:3450–3458.
16. Farrell TG, Odemuyiwa O, Bashir Y, Gripps TR, Malik M, Ward DE, Camm AJ. Prognostic value of baroreflex sensitivity testing after acute myocardial infarction. *Br Heart J* 1992;67: 129–137.
17. Hesse C, Charkoudian N, Liu Z, Joyner MJ, Eisenach JH. Baroreflex sensitivity inversely correlates with ambulatory blood pressure in healthy normotensive humans. *Hypertension* 2007;50: 41–46.
18. Imholz BP, Wielong W, Langewouters GJ, van Montfrans GA. Continuous finger arterial pressure: utility in the cardiovascular laboratory. *Clin Auton Res* 1991;l1:43–53.
19. Imholz BP, Wieling W, van Montfrans GA, Wesseling KH. Fifteen years experience with finger arterial pressure monitoring: assessment of the technology. *Cardiovasc Res* 1998;38:605–616.
20. Waki H, Kasparov S, Wong LF, Murphy D, Shimizu T, Paton JFR. Chronic inhibition of eNOS activity in nucleus tractus solitarii enhances baroreceptor reflex in conscious rats. *J Physiol* 2003;546:233–242.
21. Waki H, Katahira K, Polson JW, Kasparov S, Murphy D, Paton JFR. Automation of analysis of cardiovascular autonomic function from chronic measurements of arterial pressure in conscious rats. *Exp Physiol*. 2006;91:201–213.
22. Gillman MW, Kannel WB, Belanger A, D'Agostuno RB. Influence of heart rate on mortality among patients with hypertension: the Framingham Study. *Am Heart J* 1993;125:1148–1154.
23. Furberg CD, Psaty BM, Meyer JV. Nifedipine: Dose-related increase in mortality in patients with coronary heart disease. *Circulation* 1995;92:1326–1331.
24. Psaty BM, Heckbert SR, Koepsell TD. The risk of myocardial infarction associated with antihypertensive drug therapies. *JAMA* 1995;274:620–625.

25. Johnson P, Shore A, Potter J, Panerai R, James M. Baroreflex sensitivity measured by spectral and sequence analysis in cerebrovascular disease. *Clin Auton Res* 2006;16:270–275.
26. Kishi T, Hirooka Y, Kimura Y, Sakai K, Ito K, Shimokawa H, Takeshita A. Overexpression of eNOS in RVLM improves impaired baroreflex control of heart rate in SHRSP. *Hypertension* 2003;41:255–260.
27. Eguchi K, Tomizawa H, Ishikawa J, Hoshide S, Pickering TG, Shimada K, Kario K. Factors associated with baroreflex sensitivity: associated with morning blood pressure. *Hypertens Res* 2007;30:723–728.
28. Milan A, Caserta MA, Del Colle S, Dematteis A, Morello F, Rabbia F, Mulatero P, Pandian NG, Veglio F. Baroreflex sensitivity correlates with left ventricular morphology and diastolic function in essential hypertension. *J Hypertens* 2007;25:1655–1664.
29. Eguchi K, Tomizawa H, Ishikawa J, Hoshide S, Fukuda T, Numao T, Shimada K, Kario K. Effects of new calcium channel blocker, azelnidipine, and amlodipine on baroreflex sensitivity and ambulatory blood pressure. *J Cardiovasc Pharmacol* 2007;49:394–400.
30. Yamagishi T. Beneficial effect of cilnidipine on morning hypertension and white-coat effect in patients with essential hypertension. *Hypertens Res* 2006;29:339–344.
31. Sakaki T, Naruse H, Masai M. Cilnidipine as an agent to lower blood pressure without sympathetic nervous activation as demonstrated by iodine-123 metaiodobenzyl guanidine imaging in rat heart. *Ann Nucl Med* 2003;17:321–326.
32. Sakata K, Yoshida H, Obayashi K. Comparative effect of cilnidipine and quinapril on left ventricular mass in mild essential hypertension. *Drugs Exp Clin Res* 2003;29:117–123.
33. Nagai H, Minatoguchi S, Chen XH. Cilnidipine, an N+L-type dihydropyridine Ca channel blocker, suppresses the occurrence of ischemia/reperfusion arrhythmia in a rabbit model of myocardial infarction. *Hypertens Res* 2005;28:361–368.

Therapeutic Neovascularization by Nanotechnology-Mediated Cell-Selective Delivery of Pitavastatin Into the Vascular Endothelium

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Objective—Recent clinical studies of therapeutic neovascularization using angiogenic growth factors demonstrated smaller therapeutic effects than those reported in animal experiments. We hypothesized that nanoparticle (NP)-mediated cell-selective delivery of statins to vascular endothelium would more effectively and integratively induce therapeutic neovascularization.

Methods and Results—In a murine hindlimb ischemia model, intramuscular injection of biodegradable polymeric NP resulted in cell-selective delivery of NP into the capillary and arteriolar endothelium of ischemic muscles for up to 2 weeks postinjection. NP-mediated statin delivery significantly enhanced recovery of blood perfusion to the ischemic limb, increased angiogenesis and arteriogenesis, and promoted expression of the protein kinase Akt, endothelial nitric oxide synthase (eNOS), and angiogenic growth factors. These effects were blocked in mice administered a nitric oxide synthase inhibitor, or in eNOS-deficient mice.

Conclusions—NP-mediated cell-selective statin delivery may be a more effective and integrative strategy for therapeutic neovascularization in patients with severe organ ischemia. **(*Arterioscler Thromb Vasc Biol.* 2009;29:796-801.)**

Key Words: nanotechnology ■ drug delivery system ■ statin ■ therapeutic neovascularization

Restoration of tissue perfusion in patients with critical ischemia attributable to coronary artery disease and peripheral artery disease is a major therapeutic goal. Recently, double-blind placebo-controlled clinical trials designed to induce neovascularization by administering exogenous angiogenic growth factors failed to demonstrate a clinical benefit and produced some undesired side effects.[1,2] These nonoptimal clinical results were in contrast to the results obtained in animal experiments and small open-label clinical trials.[3,4] The disappointing results of the clinical trials of therapeutic angiogenesis may be attributable in part to less effective transfection of the genetic materials or the rapid washout of proteins. In addition, because the involvement of multiple endogenous angiogenic growth factors is required for the development of functional collaterals,[5,6] the strategy of simple intramuscular injection of an exogenous angiogenic growth factor is limited. A high local concentration of angiogenic growth factors increases the risks of edema,[3,7] angioma-like capillary formation,[7–9] atherosclerosis after vascular injury,[10–13] and tumor-angiogenesis.[7,8] A controlled drug delivery system (DDS) for an integrative approach to therapeutic neovascularization would be more favorable.

To address this challenge, we developed a novel nanoparticle (NP)-mediated DDS, formulated from the bioabsorbable polylactide/glycolide copolymer (PLGA).[14] The PLGA NP offers the advantages of safety, delivery of encapsulated drugs into the cellular cytoplasm, and slow cytoplasmic drug release.[14,15] PLGA NP are effectively and rapidly taken up by vascular endothelial cells in vitro.[16] To our knowledge, however, no prior studies have examined whether PLGA NPs are useful as an endothelial cell-selective DDS in vivo.

We hypothesized that HMG-CoA reductase inhibitors, so-called statins, are appropriate candidate drugs for this integrative approach, because statins have a variety of pleiotropic vasculoprotective effects that are independent of their lipid-lowering activity.[17] Statins increase the angiogenic activity of mature endothelial cells as well as that of endothelial progenitor cells (EPCs)[18,19] and augment collateral growth in ischemic heart and limb in experimental animals.[20,21] In addition, statins attenuate atherosclerosis formation[22,23] and have little potential risk of tumor angiogenesis in contrast to angiogenic growth factors.[24] Most of these beneficial effects of statin on therapeutic neovascularization, however, were observed after daily administration of high doses,[18–21] which

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may lead to serious adverse side effects in a clinical setting. Because vascular endothelium plays a primary role in the pathogenesis of ischemia-induced neovascularization, we hypothesized that NP-mediated cell-selective delivery of statins to the vascular endothelium would more effectively and integratively induce therapeutic neovascularization.

The major aim of this study was to test the hypothesis that selective NP-mediated delivery of statins to endothelial cells can be an integrative approach to enhance therapeutic neovascularization. We used a murine model of hindlimb ischemia to examine, (1) whether PLGA NPs are delivered selectively to vascular endothelial cells in ischemic tissues; and (2) whether NP-mediated delivery of statin is useful for increasing therapeutic neovascularization.

Materials and Methods

Preparation of PLGA NPs

Anionic PLGA NPs encapsulated with fluorescein isothiocyanate (FITC) or pitavastatin were prepared by a previously reported emulsion solvent diffusion method in purified water. The diameter of the PLGA NPs was 196±29 nm. The PLGA NPs had a negative surface charge (-15±10 mV). The FITC- and pitavastatin-loaded PLGA NPs contained 5% (wt/vol) FITC and 5% (wt/vol) pitavastatin, respectively. Additional details are provided in the supplemental information (please see http://atvb.ahajournals.org.).

Intracellular Uptake and Intracellular Distribution of NPs

Human umbilical vein endothelial cells (HUVECs) were obtained and cultured in EGM-2. Human skeletal muscle cells (SkMCs) were obtained and cultured in SkGM. Additional details can be found in the supplemental information.

Angiogenesis Assay of Human Endothelial Cells

Angiogenesis assay of human endothelial cells was tested using a 2-dimensional Matrigel assay. Additional details are provided in the supplemental information.

Animal Preparation and Experimental Protocol

Male 8-week-old C57BL/6J wild-type mice were used. After anesthesia, we induced unilateral hindlimb ischemia in the mice as previously described.[25] Immediately after the induction of ischemia, animals were randomly divided into 4 groups; a control no treatment group and the remaining groups received intramuscular injections of FITC-NPs (NP group), pitavastatin at 0.4 mg/kg (statin only group), or pitavastatin-NPs containing 0.4 mg/kg pitavastatin (statin-NP group) into the left femoral and thigh muscles. Biochemical parameters listed in supplemental Table I were measured 3, 7, and 14 days after treatment. Additional details are provided in the supplemental information.

Limb blood flow measurements were performed using a laser Doppler perfusion imaging (LDPI) analyzer (Moor Instruments). The LDPI index was expressed as the ratio of the LDPI signal in the ischemic limb compared to that in the normal limb.[25]

Histological and Immunohistochemical Analyses

Histological and immunohistochemical evaluation was performed. To determine capillary and arteriolar density, cross sections were stained with anti-mouse platelet endothelial cell adhesion molecule (PECAM)-1 antibody (CD31) and α-smooth muscle actin (α-SMA), respectively. Additional details are provided in the supplemental information.

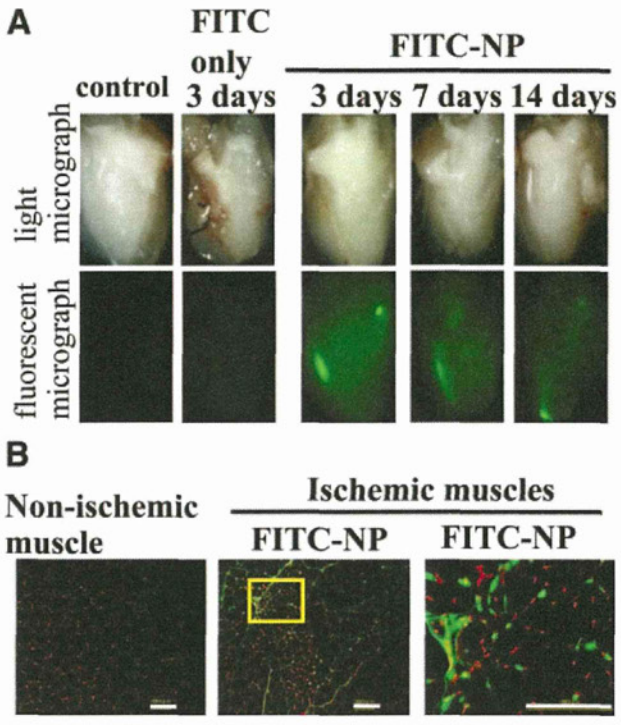

Figure 1. A, Representative light and fluorescent stereomicrographs of gastrocnemius muscles from control nonischemic hindlimb and from ischemic hindlimb. B, Fluorescent micrographs of cross-sections from nonischemic muscle with no injection, ischemic muscles 14 days after the injection of FITC-NP, and expanded view of boxed area of middle panel. Scale bars: 100 μm.

Western Blotting

Protein expression of Akt, eNOS, VEGF, FGF-2, and MCP-1 was examined 7 days after the induction of hindlimb ischemia. Additional details are provided in the supplemental information.

Flow Cytometric Analyses of EPC Mobilization

Peripheral blood was obtained from mice 7 and 14 days after hindlimb ischemia and analyzed with a FACS Caliber flow cytometer (Becton Dickinson). Additional details are provided in the supplemental Information.

Measurements of Statin Concentration in Serum and Muscle Tissue

Statin concentration in serum and muscle were measured at predetermined time points using a column-switching high performance liquid chromatography system. Additional details are provided in the supplemental information.

Statistical Analysis

Data are expressed as means±SEM. The statistical analysis was assessed using analysis of variance and multiple comparison tests. Probability values less than 0.05 were considered to be statistically significant.

Results

Cell-Selective Delivery of NPs In Vivo

Cellular distribution of FITC was examined 3, 7, and 14 days after intramuscular injection of FITC-NP or FITC only. On day 3 postinjection, strong FITC signals were detected only in FITC-NP injected ischemic muscle, whereas no FITC signals were observed in control nonischemic muscle or in ischemic muscle injected with FITC only (Figure 1A). The FITC

signals were localized predominantly in the capillaries and arterioles. FITC signals were also detected in myocytes at this time point. These data suggest that NP solution might distribute to intra- and extracellular spaces of ischemic skeletal muscle tissues immediately after intramuscular injection of NPs, and then the NP was uptaken by cells in injected muscles (endothelial cells, smooth muscle cells, myocytes, etc) or retained in extracellular spaces at this early time point.

On days 7 and 14, FITC signals remained localized predominantly in capillaries and arterioles (Figure 1B). Immunofluorescent staining revealed FITC signals localized mainly in endothelial cells positive for CD31, a marker of angiogenesis, in FITC-NPs injected ischemic muscle 14 days postischemia (supplemental Figure I). In contrast, no FITC signals were observed in myocytes. FITC signals were not detected in contralateral nonischemic hindlimb or in remote organs (liver, spleen, kidney, and heart) at any time point (data not shown).

Cellular Delivery of NPs Into Vascular Endothelial Cells Versus Skeletal Myocytes In Vitro

Cellular uptake of NPs was examined in HUVECs and SkMCs after incubation with FITC-NPs for 1 hour. The number of FITC-positive cells was greater among HUVECs than among SkMCs (supplemental Figure IIA). An inhibitor of clathrin-mediated endocytosis, chlorpromazine (CPZ), did not affect the magnitude of cellular FITC signals in SkMCs, but reduced the magnitude in HUVECs (supplemental Figure IIB). Long-term cell culture after 1-hour incubation with FITC-NPs revealed cellular FITC signals in HUVECs on days 3 and 7 postincubation (supplemental Figure IIC). In contrast, no FITC signal was detected in SkMC (data not shown).

Effects of Statin-NP on Ischemia-Induced Neovascularization

Treatment with statin-NP that contains pitavastatin at 0.4 mg/kg, but not with FITC-NP or statin only, significantly increased blood flow recovery on days 7 and 14 (Figure 2A and 2B). The beneficial effects of statin-NP were not associated with significant changes in serum biochemical markers (supplemental Table I), but angiogenesis and arteriogenesis were significantly increased (Figure 2C). Examination of hematoxylin-eosin-stained sections revealed no abnormal histopathologic findings (inflammation and fibrosis) among the 4 groups (data not shown). There was no significant difference in muscle fiber density among the 4 groups (data not shown).

Single intramuscular injection of nonnanoparticulated soluble pitavastatin at doses of 4 and 20 mg/kg exerted no effect on blood perfusion after hindlimb ischemia (supplemental Figure IIIA). Oral daily administration of pitavastatin at 0.4 mg/kg did not increase blood flow recovery, but pitavastatin at 1.0 and 10 mg/kg significantly increased blood flow recovery on day 14 (supplemental Figure IV).

Systemic daily administration of statins is reported to increase EPC mobilization,[18,26] but the EPC number in the circulating blood was not increased in the present study (supplemental Figure IIIB and IIIC). No therapeutic effects of

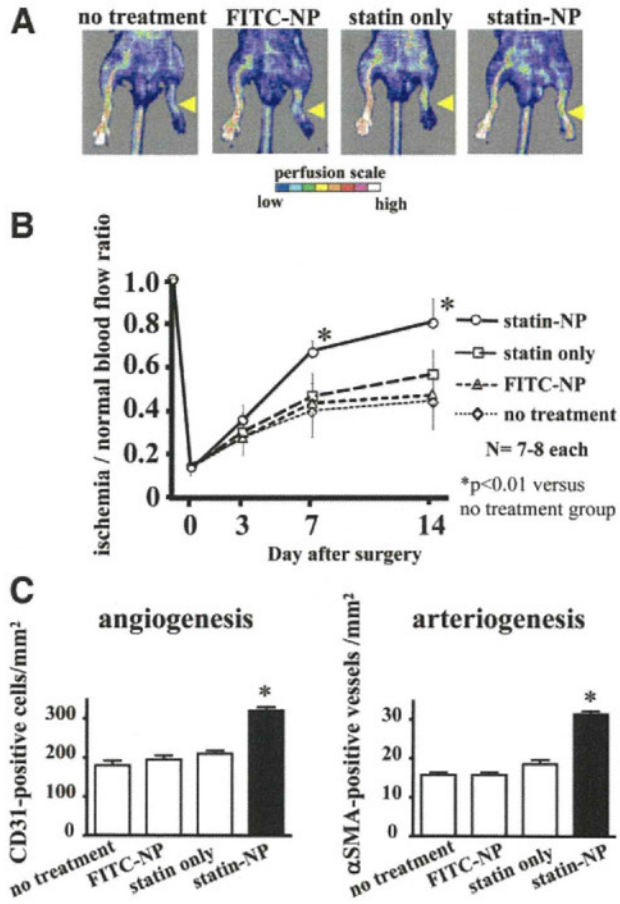

Figure 2. A, Representative laser Doppler perfusion imaging at 14 days postischemia. Arrow indicates ischemic limb. B, Quantification of blood flow recovery. C, Quantitative analysis of angiogenesis and arteriogenesis. n=4 each. *$P<0.01$ vs no treatment group.

statin-NP were observed in wild-type mice administered L-NAME or in eNOS$^{-/-}$ mice (Figure 3A), suggesting that eNOS-related signals are involved in the mechanism of statin-induced enhancement of ischemia-induced neovascularization (supplemental Figure V). Treatment with statin-NP increased both phosphorylated eNOS and serine-threonine specific protein kinase (Akt) in ischemic muscles compared with nonischemic control and nontreated ischemic muscles at 7 days of treatment (Figure 3B). Immunohistochemistry revealed that the increased eNOS and Akt activities were localized mainly in microvascular endothelial cells (supplemental Figure VI).

Effect of Statin-NP on Endogenous Angiogenic Growth Factor Expression

Immunohistochemistry was performed to examine the cellular localization of angiogenic growth factors in control and statin-NP groups. On day 3, immunostaining for both vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) was observed in skeletal myocytes and blood vessels (supplemental Figure VII). On days 7 and 14, the immunostaining intensity markedly decreased in skeletal myocytes and blood vessels in the control group. In contrast, positive immunostaining was observed in endothelial cells of

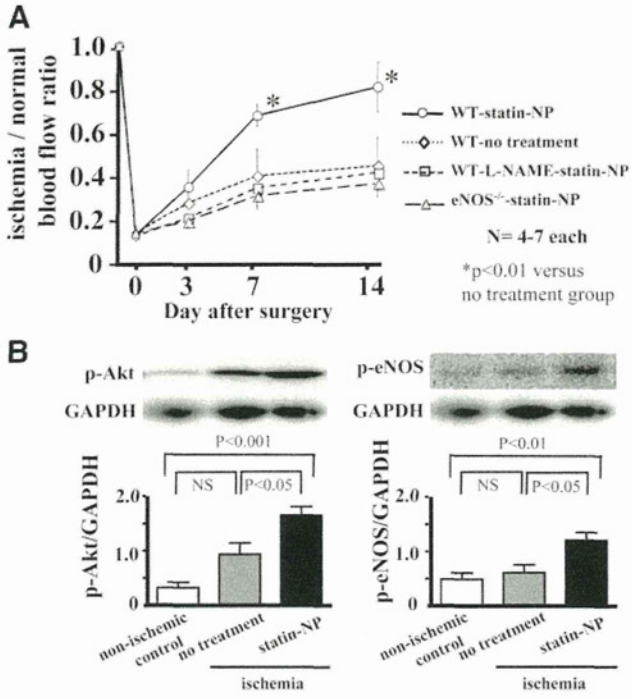

Figure 3. A, Quantification of blood flow recovery in wild-type (WT) mice with or without administration of L-NAME, a NOS inhibitor, and in $eNOS^{-/-}$ mice. B, Western blot analysis of phosphorylated Akt and eNOS in ischemic and nonischemic muscles 7 days after ischemia. n=6 each. NS=not significant.

capillaries and arterioles in the statin-NP group on days 7 and 14. Western blot analysis revealed greater protein expression of VEGF, FGF-2, and monocyte chemotactic protein-1 (MCP-1) in ischemic muscle in the statin-NP group than in the no treatment group 7 days after hindlimb ischemia (Figure 4). Interestingly, the increased expression of such angiogenic growth factors by treatment with statin-NP was blunted in mice administered chronically with L-NAME.

Effects of Statin-NP on Angiogenic Capacity of Human Endothelial Cells In Vitro

Cotreatment with statin or statin-NP increased angiogenic activity in HUVECs. The angiogenic activity of statin-NP was greater than that of 10 nmol/L statin only (supplemental Figure VIIIA). Pretreatment with statin only (24-hour incubation of HUVECs with statin) had no angiogenic effects at any dose. In contrast, pretreatment with statin-NP induced significant angiogenic effects at 1 and 10 nmol/L compared with the no-treatment control group (supplemental Figure VIIIB).

Serum and Tissue Concentrations of Statin

Tissue concentrations of pitavastatin were greater in skeletal muscles injected with statin-NPs than in those injected with statin 6 and 24 hours after intramuscular administration, whereas serum levels of pitavastatin were comparable between the 2 groups (supplemental Table II). The drug was not detected in serum 1 and 3 days after injection.

Discussion

The application of nanotechnology-based drug delivery is expected to have a major impact on the development of

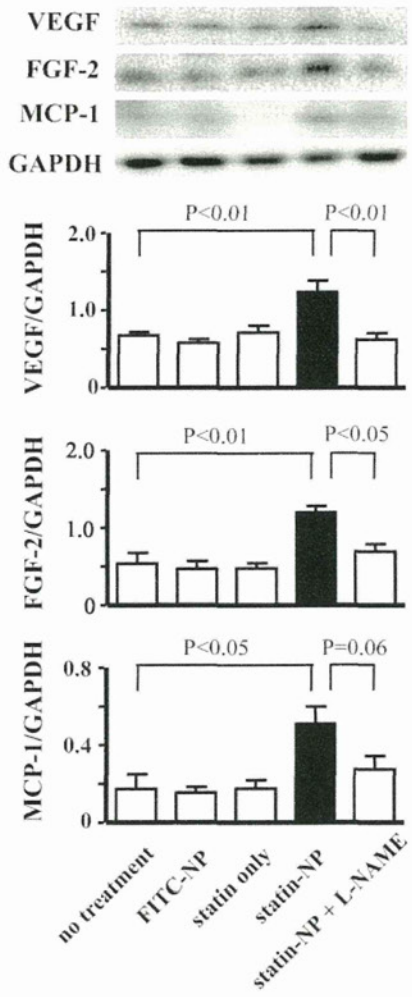

Figure 4. Effects of statin-NP on expression of VEGF, FGF-2, and MCP-1 in ischemic muscle. Densitometric analysis of protein expression in ischemic muscles 7 days after ischemia. Quantitative evaluation was expressed as a ratio of VEGF, FGF-2, and MCP-1 to GAPDH. n=6 each.

innovative medicines. In the present study, selective NP-mediated delivery of statin to vascular endothelial cells increased neovascularization and improved tissue perfusion in a murine model of hindlimb ischemia, indicating that this novel cell-selective delivery system is feasible for therapeutic neovascularization.

The most novel finding of this study is that FITC signals were localized mainly in the vascular endothelium 7 and 14 days after injection of FITC-NP into ischemic skeletal muscles in vivo. Several factors might be involved in mechanisms of the cell-selective delivery of the NP at later time points. First, increased endocytosis of NP in the endothelium may be involved, which is based on our present experiments with CPZ, an inhibitor of clathrin-mediated endocytosis. In addition, 1-hour incubation with FITC-NP resulted in long-term and stable retention of NP in the human endothelial cells, but not in skeletal myocytes in vitro. Second, decreased exocytosis of the endothelium in the presence of ischemia might also be involved. Third, after cellular delivery of NP via endocyotosis, rapid escape of the NP from the endosomal compartment to the cytoplasmic compartment may lead to

sustained intracellular drug delivery and good efficacy. The NP is likely retained in the cytoplasm where release of the encapsulated drug occurs slowly in conjunction with the hydrolysis of PLGA.[15] Overall, the nanotechnology platform for cell-selective delivery to the vascular endothelium using NP may be useful as an innovative strategy for therapeutic neovascularization and other intractable diseases.

Another important feature of this study is that a single administration of statin-NP containing pitavastatin (0.4 mg/kg) into vascular endothelial cells effectively increased therapeutic neovascularization with no serious side effect in murine model of hindlimb ischemia. Sata et al[24] reported that systemic daily administration of pitavastatin (1 mg/kg per day × 49 days=49 mg/kg) has significant therapeutic effects in mice with hindlimb ischemia. In the present study, we confirmed the study of Sata et al[24] by showing that oral daily administration of pitavastatin for 14 days (1 and 10 mg/kg per day × 14=14 and 140 mg/kg, respectively) had significant therapeutic effects, as did statin-NP (0.4 mg/kg). Therefore, our NP-mediated delivery system seems to be as effective at an approximately 100-times lower dose than the cumulative systemic dose. Furthermore, measurement of the tissue and serum concentrations of pitavastatin confirmed the effective local retention of statin-NPs in ischemic skeletal muscles in vivo. NP-mediated delivery of pitavastatin accelerated angiogenic activity of human endothelial cells in vitro. Therefore, it is possible that after NP-mediated endothelial delivery, pitavastatin was slowly released from the NPs into the cytoplasm along with PLGA hydrolysis, resulting in significant therapeutic effects.

Clearly, the therapeutic neovascularization induced by statin-NPs resulted from the pleiotropic effects, because pitavastatin-NPs had no effect on serum lipid levels. Our experiments with mice treated with a NOS inhibitor and $eNOS^{-/-}$ mice support the essential role of the eNOS pathway in the mechanism underlying the therapeutic effects of NP-mediated cell-selective delivery of statin. Consistent with the results of other investigators,[18,20,21,26] we demonstrated that pitavastatin-NP increased the activity of vascular eNOS and PI3K/Akt (as shown in supplemental Figure V) in association with an increased expression of endogenous multiple angiogenic growth factors that are involved in angiogenesis (VEGF) and arteriogenesis (FGF-2, MCP-1).[27] These therapeutic effects afforded by the NP-mediated cell-selective delivery of statin were not associated with a further increase in circulating EPC. Intramuscular injection of soluble pitavastatin alone at high doses (4 and 20 mg/kg) has no therapeutic effect, suggesting a specific advantage of endothelial cell selective delivery of pitavastatin by the PLGA NP formulation. These findings suggest that pitavastatin-NP acted locally on ischemic vascular endothelium to induce therapeutic neovascularization and are consistent with the notion that NP-mediated endothelial cell-selective delivery of statin produces a well-harmonized integrative system to form functionally mature collaterals via controlled expression of endogenous multiple angiogenic growth factors and signals, allowing for a more effective model for an integrative approach to therapeutic neovascularization.

There is a major limitation to the present study. First, we examined only a single dose of statin-NPs. It is difficult to obtain a dose-response relationship of this NP system in small animals. For translation of our present findings into clinical medicine, further studies are needed to define the dose-response relation in large animal models. This point is important because statins are reported to exert a double-edged role in angiogenesis signaling.[28] Although such antiangiogenic effects of statins at high dose did not occur in a murine model,[24] this must be examined in large animal models. Second, we only examined the therapeutic effects of a single intramuscular injection of statin-NP. Whether repetitive delivery of statin-NP at an optimal dose over time produces greater therapeutic effects remains to be investigated.

In conclusion, this platform nanotechnology of vascular endothelial cell-selective delivery of statin is a promising strategy toward more effective and integrative nanomedicine in patients with severe organ ischemia, and represents a significant advance in therapeutic neovascularization over current approaches. The nanotechnology platform may be developed further as an "integrative" approach for therapeutic neovascularization, and extended to target other molecular signals specific to vascular endothelial cells.

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We thank Eiko Iwata and Miho Miyagawa for their technical supports in this study.

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Disclosures

Dr Egashira holds a patent on the results reported in the present study. The remaining authors report no conflicts.

References

1. Losordo DW, Dimmeler S. Therapeutic angiogenesis and vasculogenesis for ischemic disease. Part I: angiogenic cytokines. *Circulation*. 2004;109:2487–2491.
2. Losordo DW, Dimmeler S. Therapeutic angiogenesis and vasculogenesis for ischemic disease: part II: cell-based therapies. *Circulation*. 2004;109:2692–2697.
3. Baumgartner I, Pieczek A, Manor O, Blair R, Kearney M, Walsh K, Isner JM. Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation*. 1998;97:1114–1123.
4. Marui A, Tabata Y, Kojima S, Yamamoto M, Tambara K, Nishina T, Saji Y, Inui K, Hashida T, Yokoyama S, Onodera R, Ikeda T, Fukushima M, Komeda M. A novel approach to therapeutic angiogenesis for patients with critical limb ischemia by sustained release of basic fibroblast growth factor using biodegradable gelatin hydrogel: an initial report of the phase I-IIa study. *Circ J*. 2007;71:1181–1186.
5. Schaper W, Scholz D. Factors regulating arteriogenesis. *Arterioscler Thromb Vasc Biol*. 2003;23:1143–1151.
6. Heil M, Schaper W. Influence of mechanical, cellular, and molecular factors on collateral artery growth (arteriogenesis). *Circ Res*. 2004;95:449–458.
7. Ferrara N, Alitalo K. Clinical applications of angiogenic growth factors and their inhibitors. *Nat Med*. 1999;5:1359–1364.
8. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature*. 2000;407:249–257.

9. Yonemitsu Y, Kaneda Y, Morishita R, Nakagawa K, Nakashima Y, Sueishi K. Characterization of in vivo gene transfer into the arterial wall mediated by the Sendai virus (hemagglutinating virus of Japan) liposomes: an effective tool for the in vivo study of arterial diseases. *Lab Invest*. 1996;75:313–323.
10. Ohtani K, Egashira K, Hiasa K, Zhao Q, Kitamoto S, Ishibashi M, Usui M, Inoue S, Yonemitsu Y, Sueishi K, Sata M, Shibuya M, Sunagawa K. Blockade of vascular endothelial growth factor suppresses experimental restenosis after intraluminal injury by inhibiting recruitment of monocyte lineage cells. *Circulation*. 2004;110:2444–2452.
11. Zhao Q, Egashira K, Hiasa K, Ishibashi M, Inoue S, Ohtani K, Tan C, Shibuya M, Takeshita A, Sunagawa K. Essential role of vascular endothelial growth factor and Flt-1 signals in neointimal formation after periadventitial injury. *Arterioscler Thromb Vasc Biol*. 2004;24:2284–2289.
12. Zhao Q, Egashira K, Inoue S, Usui M, Kitamoto S, Ni W, Ishibashi M, Hiasa Ki K, Ichiki T, Shibuya M, Takeshita A. Vascular endothelial growth factor is necessary in the development of arteriosclerosis by recruiting/activating monocytes in a rat model of long-term inhibition of nitric oxide synthesis. *Circulation*. 2002;105:1110–1115.
13. Celletti FL, Waugh JM, Amabile PG, Brendolan A, Hilfiker PR, Dake MD. Vascular endothelial growth factor enhances atherosclerotic plaque progression. *Nat Med*. 2001;7:425–429.
14. Kawashima Y, Yamamoto H, Takeuchi H, Hino T, Niwa T. Properties of a peptide containing DL-lactide/glycolide copolymer nanospheres prepared by novel emulsion solvent diffusion methods. *Eur J Pharm Biopharm*. 1998;45:41–48.
15. Panyam J, Zhou WZ, Prabha S, Sahoo SK, Labhasetwar V. Rapid endolysosomal escape of poly(DL-lactide-co-glycolide) nanoparticles: implications for drug and gene delivery. *Faseb J*. 2002;16:1217–1226.
16. Davda J, Labhasetwar V. Characterization of nanoparticle uptake by endothelial cells. *Int J Pharm*. 2002;233:51–59.
17. Takemoto M, Liao JK. Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors. *Arterioscler Thromb Vasc Biol*. 2001; 21:1712–1719.
18. Llevadot J, Murasawa S, Kureishi Y, Uchida S, Masuda H, Kawamoto A, Walsh K, Isner JM, Asahara T. HMG-CoA reductase inhibitor mobilizes bone marrow–derived endothelial progenitor cells. *J Clin Invest*. 2001; 108:399–405.
19. Altieri DC. Statins' benefits begin to sprout. *J Clin Invest*. 2001;108: 365–366.
20. Sata M, Nishimatsu H, Suzuki E, Sugiura S, Yoshizumi M, Ouchi Y, Hirata Y, Nagai R. Endothelial nitric oxide synthase is essential for the HMG-CoA reductase inhibitor cerivastatin to promote collateral growth in response to ischemia. *Faseb J*. 2001;15:2530–2532.
21. Kureishi Y, Luo Z, Shiojima I, Bialik A, Fulton D, Lefer DJ, Sessa WC, Walsh K. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. *Nat Med*. 2000;6:1004–1010.
22. Kitamoto S, Nakano K, Hirouchi Y, Kohjimoto Y, Kitajima S, Usui M, Inoue S, Egashira K. Cholesterol-lowering independent regression and stabilization of atherosclerotic lesions by pravastatin and by antimonocyte chemoattractant protein-1 therapy in nonhuman primates. *Arterioscler Thromb Vasc Biol*. 2004;24:1522–1528.
23. Ni W, Egashira K, Kataoka C, Kitamoto S, Koyanagi M, Inoue S, Takeshita A. Antiinflammatory and antiarteriosclerotic actions of HMG-CoA reductase inhibitors in a rat model of chronic inhibition of nitric oxide synthesis. *Circ Res*. 2001;89:415–421.
24. Sata M, Nishimatsu H, Osuga J, Tanaka K, Ishizaka N, Ishibashi S, Hirata Y, Nagai R. Statins augment collateral growth in response to ischemia but they do not promote cancer and atherosclerosis. *Hypertension*. 2004;43: 1214–1220.
25. Hiasa K, Ishibashi M, Ohtani K, Inoue S, Zhao Q, Kitamoto S, Sata M, Ichiki T, Takeshita A, Egashira K. Gene transfer of stromal cell-derived factor-1alpha enhances ischemic vasculogenesis and angiogenesis via vascular endothelial growth factor/endothelial nitric oxide synthase-related pathway: next-generation chemokine therapy for therapeutic neovascularization. *Circulation*. 2004;109:2454–2461.
26. Dimmeler S, Aicher A, Vasa M, Mildner-Rihm C, Adler K, Tiemann M, Rutten H, Fichtlscherer S, Martin H, Zeiher AM. HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. *J Clin Invest*. 2001;108:391–397.
27. Fujii T, Yonemitsu Y, Onimaru M, Tanii M, Nakano T, Egashira K, Takehara T, Inoue M, Hasegawa M, Kuwano H, Sueishi K. Nonendothelial mesenchymal cell-derived MCP-1 is required for FGF-2-mediated therapeutic neovascularization: critical role of the inflammatory/arteriogenic pathway. *Arterioscler Thromb Vasc Biol*. 2006;26: 2483–2489.
28. Urbich C, Dernbach E, Zeiher AM, Dimmeler S. Double-edged role of statins in angiogenesis signaling. *Circ Res*. 2002;90:737–744.

Supplement Data

Therapeutic Neovascularization by Nanotechnology-Mediated Cell-Selective Delivery of Pitavastatin into the Vascular Endothelium

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Supplementary Table 1.

Serum biochemical profiles 3, 7 and 14 days after hindlimb ischemia

	No treatment	FITC-NP	Statin only	Statin-NP	*P* value
Creatine Phosphokinase (IU/L)					
day 3	105±16	144±25	138±26	141±29	0.66
day 7	75±12	100±14	83±16	65±10	0.35
day 14	76±13	56±7	74±3	52±17	0.39
Myoglobin (ng/ml)					
day 3	< 10	< 10	< 10	< 10	-
day 7	< 10	< 10	< 10	< 10	-
day 14	< 10	< 10	< 10	< 10	-
AST (IU/L)					
day 3	63±10	54±24	64±14	60±16	0.97
day 7	34±1	37±6	47±8	32±7	0.37
day 14	35±7	29±2	38±5	35±3	0.57
ALT (IU/L)					
day 3	27±5	24±1	22±3	27±8	0.86
day 7	13±1	23±10	18±3	15±4	0.64
day 14	19±1	17±0	24±5	22±3	0.35
BUN (mg/dl)					
day 3	26±2.5	29±1.5	30±0.3	29±3.6	0.75
day 7	28±1	23±5	22±1	22±1	0.44
day 14	33±2	34±2	35±1	37±1	0.36
Creatinine (mg/dl)					
day 3	0.11±0.01	0.10±0.01	0.14±0.01	0.14±0.02	0.11
day 7	0.10±0.01	0.10±0.01	0.11±0.01	0.10±0.01	0.69
day 14	0.12±0.01	0.11±0.02	0.10±0.01	0.10±0.01	0.50
Total cholesterol (mg/dl)					
day 3	95±3	102±1	90±2	105±12	0.40
day 7	87±4	92±11	81±4	76±3	0.39
day 14	76±2	84±5	87±5	80±2	0.25
LDL cholesterol (mg/dl)					
day 3	24±2	22±2	24±3	27±5	0.72
day 7	12±2	21±9	15±2	13±2	0.56
day 14	5±1	5±0	8±1	7±2	0.33

Data are mean±SEM (n=3 each)

Supplementary Table 2.

Tissue and serum pitavastatin concentrations after intramuscular injection of statin

	time after injection		
	6 hours	1 day	3 day
Pitavastatin at 0.4 mg/kg			
muscle (ng/g tissue)	305 ± 80	81 ± 60	4 ± 3
serum (ng/ml)	3 ± 0.3	ND	ND
Statin-NP containing 0.4 mg/kg of pitavastatin			
muscle (ng/g tissue)	2088 ± 412*	692 ± 288*	9 ± 5
serum (ng/ml)	5 ± 0.7	ND	ND

Data are mean±SEM (n=8 to 9 each). ND: not detected. *P<0.01 versus intramuscular pitavastatin.

Supplementary Figure Legends

Supplementary Figure I. Immunofluorescent staining of cross-sections from ischemic muscle 14 days after FITC-NP injection stained with an endothelial marker, CD31 (red). Inset left below is non-injected control muscle. Scale bars: 100 μm.

Supplementary Figure II. Cell-selective delivery of FITC-NP into vascular endothelial cells versus skeletal myocytes. A, Fluorescent micrographs of HUVEC and SkMC incubated with FITC-NP (0.1 mg/ml) for 1 hour and percentage of FITC-positive cells (n=5 each). Nuclei were counter stained with PI. Scale bars: 100 μm. B, Effects of chlorpromazine (CPZ) on cellular distribution of NP in HUVEC and SkMC. Quantitative analysis of magnitude of intracellular FITC fluorescence signals in 3 independent experiments are shown. *$p<0.05$, **$p<0.001$ versus control condition. C, Fluorescent micrographs of HUVEC immediately after, and 3 and 7 days after the 1 hour incubation with FITC-NPs. Inset left above is control HUVEC without FITC-NP. Nuclei were counter stained with PI. Scale bars: 100 μm.

Supplementary Figure III. A, Quantification of LDPI–derived blood flow recovery expressed as the ratio of ischemic to normal limb at 7 and 14 days. Mice were injected with pitavastatin at 4 and 20 mg/kg into the ischemic muscle immediately after induction of hindlimb ischemia. N = 5 to 6. NS=no significance. B, Representative scatter diagram of Sca-1/Flk-1-double positive EPCs in peripheral blood analyzed by flow cytometry 14 days after induction of hindlimb ischemia (circled region). C, Quantitative analysis of circulating EPCs expressed as percentage of Sca-1/Flk-1 double positive cells to total leukocytes 7 and 14 days after ischemia. N = 4 to 5 each. *$p<0.05$, **$p<0.001$ versus non-ischemic control group.

Supplementary Figure IV. Effects of oral daily administration of pitavastatin on ischemia-induced neovascularization. Quantification of laser Doppler perfusion imaging (LDPI)–derived blood flow recovery at 14 days. n=6 to 8. *$p<0.05$, **$p<0.01$ versus no treatment group.

Supplementary Figure V. Schematic illustration of the effects of statins on intracellular pathways. Mevalonate, the end product of the HMG-CoA, inhibits PI3K and the subsequent phosphorylation of Akt and eNOS. Blockade of HMG-CoA reductase with statins is expected to result in the increase of activity of Akt and eNOS.

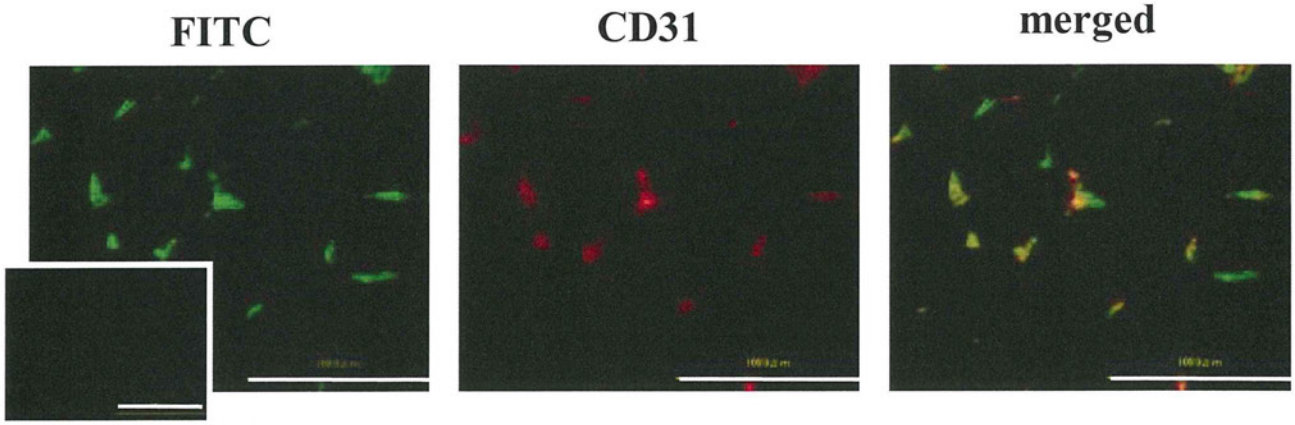
Supplementary Figure VI. Representative micrographs of ischemic muscle sections stained immunohistochemically with antibodies against phospho-Akt and phospho-eNOS at 14 days after surgery. Scale bars: 100 μm.

Supplementary Figure VII. Effects of statin-NP on the protein expression of VEGF (A) and FGF-2 (B) in ischemic muscle. Representative photographs of immunostaing of ischemic muscles 3, 7, and 14 days after hindlimb ischemia. VEGF or FGF-2 (green) is located not only within myocytes but within capillary or vascular endothelium (yellow) on day 7 and 14 in statin-NP group. Nuclei were counter stained with DAPI (blue). Scale bars: 100 μm.

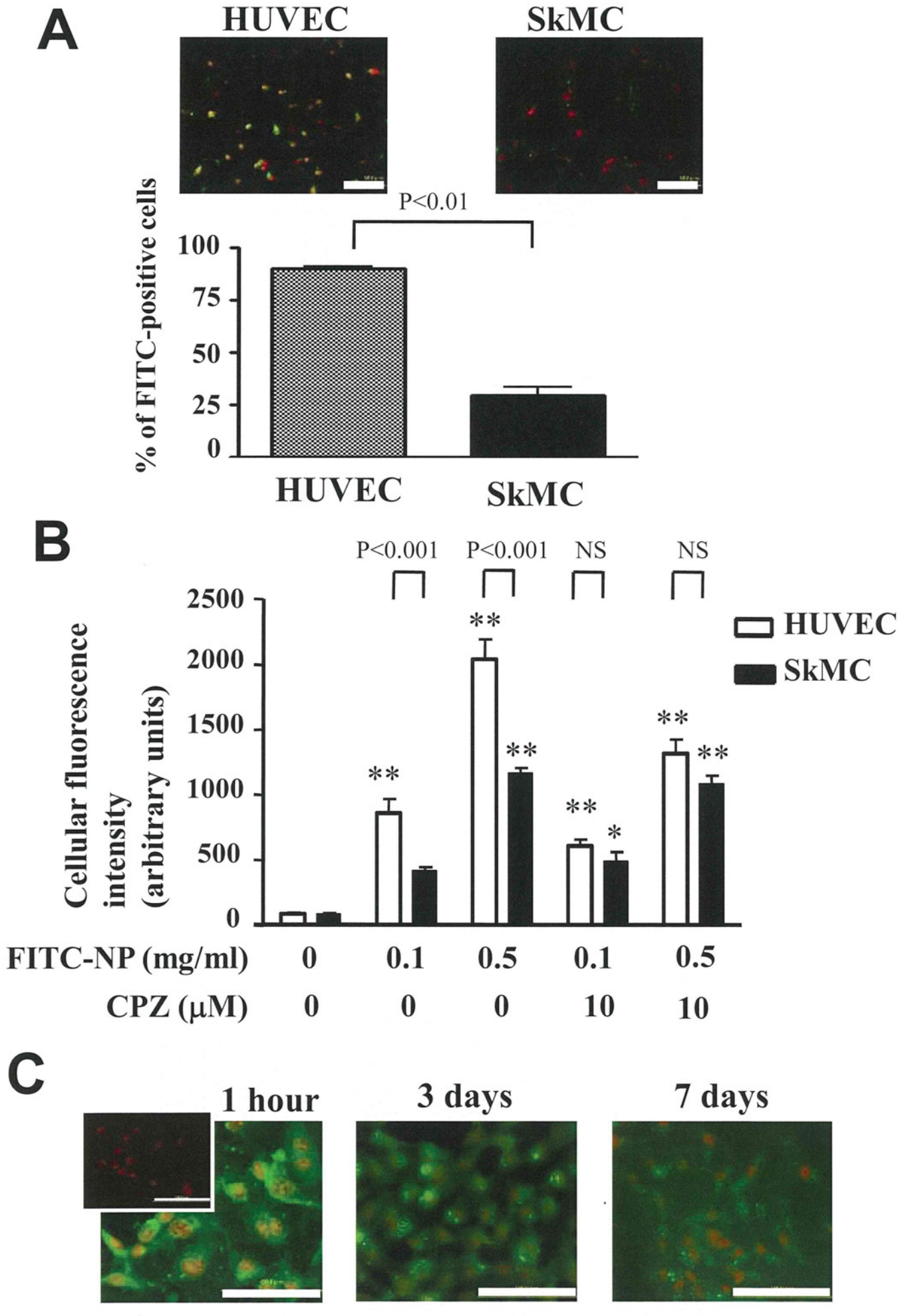
Supplementary Figure VIII. Effects of statin-NP on angiogenic capacity of human endothelial cells in vitro. A, Quantitative analysis of tube formation (tube length) of 4

independent experiments in co-treatment protocol. *$p<0.01$, **$p<0.001$ vs control. B, Quantitative analysis of tube formation (tube length) of 4 independent experiments in pre-treatment protocol. *$p<0.05$, **$p<0.001$ versus control.

Supplementary Figure I *Kubo M et al.*



Supplementary Figure II *Kubo M et al.*



Supplementary Figure III *Kubo M et al.*