Nanoparticle-mediated endothelial cell-selective delivery of pitavastatin induces functional collateral arteries (therapeutic arteriogenesis) in a rabbit model of chronic hind limb ischemia

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Objectives: **We recently demonstrated in a murine model that nanoparticle-mediated delivery of pitavastatin into vascular endothelial cells effectively increased therapeutic neovascularization. For the development of a clinically applicable approach, further investigations are necessary to assess whether this novel system can induce the development of collateral arteries (arteriogenesis) in a chronic ischemia setting in larger animals.**
Methods: **Chronic hind limb ischemia was induced in rabbits. They were administered single injections of nanoparticles loaded with pitavastatin (0.05, 0.15, and 0.5 mg/kg) into ischemic muscle.**
Results: **Treatment with pitavastatin nanoparticles (0.5 mg/kg), but not other nanoparticles, induced angiographically visible arteriogenesis. The effects of intramuscular injections of phosphate-buffered saline, fluorescein isothiocyanate (FITC)-loaded nanoparticles, pitavastatin (0.5 mg/kg), or pitavastatin (0.5 mg/kg) nanoparticles were examined. FITC nanoparticles were detected mainly in endothelial cells of the ischemic muscles for up to 4 weeks. Treatment with pitavastatin nanoparticles, but not other treatments, induced therapeutic arteriogenesis and ameliorated exercise-induced ischemia, suggesting the development of functional collateral arteries. Pretreatment with nanoparticles loaded with vatalanib, a vascular endothelial growth factor receptor (VEGF) tyrosine kinase inhibitor, abrogated the therapeutic effects of pitavastatin nanoparticles. Separate experiments with mice deficient for VEGF receptor tyrosine kinase demonstrated a crucial role of VEGF receptor signals in the therapeutic angiogenic effects.**
Conclusions: **The nanotechnology platform assessed in this study (nanoparticle-mediated endothelial cell-selective delivery of pitavastatin) may be developed as a clinically feasible and promising strategy for therapeutic arteriogenesis in patients. (J Vasc Surg 2010;52:412-20.)**

Clinical Relevance: **Restoration of tissue perfusion in patients with critical limb ischemia is a major therapeutic goal. Recent clinical trials designed to induce neovascularization by administering exogenous angiogenic growth factors or cells failed to demonstrate a decisive clinical benefit. A controlled drug delivery system for a new approach to therapeutic neovascularization therefore would be more favorable. In the present study, we applied nanoparticle-mediated delivery system and report that endothelial cell-selective delivery of pitavastatin increased the development of collateral arteries and improved exercise-induced ischemia in a rabbit model of chronic hind limb ischemia. This nanotechnology platform is a promising strategy for the treatment of patients with severe organ ischemia and represents a significant advance in therapeutic arteriogenesis over current approaches.**

The vascular endothelium is a major target for the pleiotropic (nonlipid-related) vascular protective effects of the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (statins).[1] Statins improve endothelial dysfunction[1-3] and exert multiple vascular protective properties, mainly by enhancing the activity of endothelial nitric oxide synthase. Statins increase the angiogenic activity of mature endothelial cells, as well as that of endothelial progenitor cells, and augment neovascularization (arteriogenesis, vasculogenesis, and angiogenesis) in the ischemic hearts and limbs of

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Supported by Grants-in-Aid for Scientific Research (19390216, 19650134) from the Ministry of Education, Science, and Culture, Tokyo, by Health Science Research Grants (Research on Translational Research and Nanomedicine) from the Ministry of Health, Labor, and Welfare, Tokyo; and by grants from New Energy and Industrial Technology Development Organization (NEDO), Kawasaki, Japan.
Competition of interest: Dr Egashira holds a patent on the results reported in the present study.
Additional material for this article may be found online at www.jvascsurg.org.
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0741-5214/$36.00

doi:10.1016/j.jvs.2010.03.020

experimental animals.[4-6] Statins also attenuate atherosclerosis formation[7] and pose little potential risk for tumor angiogenesis, in contrast to angiogenic growth factors.[8]

Most of these beneficial effects of statins on therapeutic neovascularization, however, were observed after the daily administration of high doses in experimental animals, a regimen that could lead to serious adverse side effects in a clinical setting. A clinical study of 500 patients with coronary artery disease reported no effects of statins within the clinical dose range on indices of functional collateral development (arteriogenesis).[9]

To optimize the therapeutic effects of statins in the induction of therapeutic neovascularization, we recently applied nanotechnology and reported that nanoparticle (NP)-mediated pitavastatin delivery into vascular endothelial cells effectively increased therapeutic neovascularization with no serious side effects in a murine model of acute hind limb ischemia.[10] The beneficial effects induced by pitavastatin-NP were mediated by increased activity of endothelial nitric oxide synthase (eNOS) and multiple endogenous angiogenic growth factors, suggesting that this NP-mediated cell-selective delivery produces a well-harmonized integrative system for therapeutic neovascularization. Importantly, this NP-mediated delivery system was as effective at a dose that is approximately 100 to 300 times lower than the cumulative systemic dose. To translate our experimental findings in the murine model of acute hind limb ischemia to clinically applicable approaches, it is desirable to determine whether NP-mediated statin delivery into vascular endothelial cells induces the development of collateral arteries (arteriogenesis) and thus restores tissue perfusion in a setting of chronic ischemia in larger animals.

Recent evidence suggests that arteriogenesis is a very important adaptive mechanism for the restoration of perfusion to critically ischemic tissue.[11] Arteriogenesis is the process whereby a preexisting arteriole from the resistance vessel class matures into an artery of the conductance vessel class, whereas angiogenesis is the process by which a sprouting capillary originates from a preexisting capillary. Vasculogenesis represents the differentiation of bone marrow-derived endothelial progenitor cells to form a primitive vasculature. The structure and molecular interactions of arteriogenesis differ from those of angiogenesis and vasculogenesis.

Contrary to conventional paradigms,[12] angiogenesis and vasculogenesis by themselves cannot replace the conductance capacity of collateral arteries in the absence of arteriogenesis.[11,13] According to the results of clinical trials, the question has been raised about whether the angiogenesis/vasculogenesis induced by single angiogenic growth factors can induce functional collateral arteries.[14,15] A high local concentration of angiogenic growth factors increases the risk of atherosclerosis[16-18] and tumor angiogenesis.[19] Therefore, an attempt to stimulate the development of functional collateral arteries through the process of arteriogenesis represents an evolution toward a new therapeutic strategy for patients with severe ischemia due to atherosclerotic vascular disease.

The primary aim of this study was to test the hypothesis that NP-mediated delivery of pitavastatin to endothelial cells can be a realistic strategy for promoting functional collateral arteries and for improving exercise-induced ischemia in a rabbit model of chronic hind limb ischemia.

MATERIALS AND METHODS

The study protocol was reviewed and approved by the Committee on Ethics in Animal Experiments, Kyushu University Faculty of Medicine. The experiments were conducted according to the Guidelines of the American Physiological Society.

Preparation of NP. Anionic poly(lactic-co-glycolic acid) (PLGA) NP incorporated with fluorescein-isothiocyanate (FITC), pitavastatin, or vatalanib[20] (an inhibitor of receptor tyrosine kinase of vascular endothelial cell growth factor [VEGF] receptors 1-3; a gift of Novartis Pharma) were prepared by a emulsion solvent diffusion method.[10] The FITC-, pitavastatin-, and vatalanib-incorporated NP contained (w/v) 5% FITC, 6.3% pitavastatin, and 6.1% vatalanib, respectively. The diameter of PLGA NP was 196 ± 29 nm. Additional details are provided in the Appendix (online only).

Angiogenesis activity of human endothelial cells. Angiogenesis of human endothelial cells (HECs) was tested by 2-dimensional Matrigel assay, as previously described.[10] Additional details are provided in the Appendix (online only).

Rabbit model of chronic hind limb ischemia and treatments. Male Japanese White rabbits were used. To induce chronic hind limb ischemia, the left femoral artery was completely excised from its proximal origin at the branchpoint of the external iliac artery to the bifurcation of the saphenous and popliteal arteries.[21,22] For intramuscular injection, drugs incorporated with or without NP were suspended in 5 mL of phosphate-buffered saline (PBS) and injected into 10 different sites in the left medial thigh muscles with a 27-gauge needle 7 days after femoral artery excision (Appendix Fig I, online only). To define the dose-response relationship of the proarteriogenic effects of pitavastatin-NP, animals were randomly divided into a PBS group and three other treatment groups that received an intramuscular injection of pitavastatin-NP containing the three different doses of pitavastatin (0.05, 0.15, and 0.5 mg/kg).

In another set of experiments, animals were randomly distributed in groups receiving intramuscular injections of PBS, pitavastatin (0.5 mg/kg), FITC-NP, or pitavastatin (0.5 mg/kg)-NP. The effect of vatalanib-NP on arteriogenesis induced by pitavastatin-NP was also examined in another set of animals treated intramuscularly with vatalanib-NP or with vatalanib-NP and pitavastatin-NP. Additional details are provided in the Appendix (on-line only).

Effects of pitavastatin-NP on collateral arterial development 28 days after treatment

Internal iliac angiography. A 4Fr end-hole infusion catheter was introduced into the right common carotid

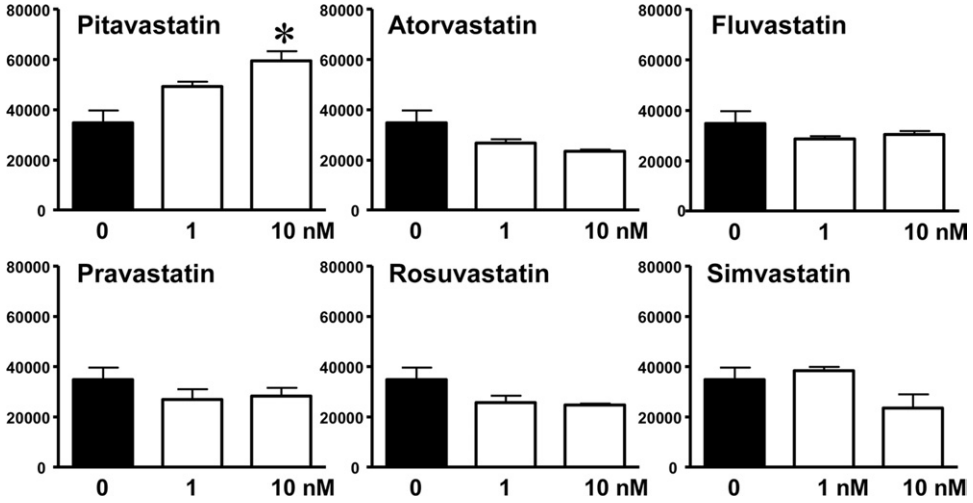

Fig 1. Effects of six statins on angiogenic capacity of human endothelial cells in vitro is shown by quantitative analysis of tube formation (tube length in mm per well) in six independent experiments. *$P < .01$ vs control by one-way analysis of variance with the Dunnett multiple comparison test.

artery and advanced to the left internal iliac artery at the level of the interspaces between the seventh lumbar and the first sacral vertebrae. After an intra-arterial injection of nitroglycerin (0.25 mg), 5 mL of contrast medium was injected at a rate of 1 mL/s. The 3-second angiogram was used for analysis of the angiographic score. A composite of 5-mm^2 grids was placed over the angiogram. The total number of grids that were crossed by visible arteries was divided by the total number of grids in the area of the medial thigh, as previously described.[21,22]

Capillary and arteriolar density. Histologic evaluation was performed for 5-μm frozen sections or 5-μm paraffin-embedded sections of the adductor skeletal muscles of the ischemic limb. CD31$^+$ (Dako, Tokyo, Japan) capillary endothelial cells were counted. Arterioles were determined by immunostaining with α-smooth muscle actin (α-SMA; Dako) and anti-mouse immunoglobulin G secondary antibody (Alexa 546; Molecular Probes, Invitrogen, Carlsbad, Calif), and vessels surrounded by smooth muscle cells were counted. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Vector Shield, Vector Laboratories, Burlingame, Calif). Capillary and arteriolar density were calculated as capillaries/mm^2 and arterioloes/mm^2 averaged from five randomly selected fields.[21,22] To ensure that the density was not overestimated or underestimated as a consequence of myocyte atrophy or edema, the capillary/muscle and arteriolar/muscle fiber ratios were also evaluated.

Tissue oximetry. Tissue oxygen content was measured by fluorescence quenching technique using an OxyLab PO_2 monitor (Oxford Optronix Ltd, Oxfordshire, UK) fiberoptic probe mounted to a micromanipulator, as previously described.[23] Ischemic limb was exposed on an anesthetized animal, and a 18-gauge needle was used to insert the fiberoptic probe to the adductor skeletal muscles of the ischemic limb at a 90° angle to contact the adductor skeletal muscles. The stable Po_2 reading, before a rapid rise to at least 60 mm Hg that signaled loss of tissue contact, was used as the tissue oxygen partial pressure.

Effects of pitavastatin-NP on forced ischemia induced by electrical pulses. The functional status of collateral arterial development was examined 28 days after treatment with PBS, FITC-NP, pitavastatin only, and pitavastatin-NP. After anesthesia, 21-gauge catheters were inserted into the right femoral artery and the left femoral vein for blood sampling. Two 21-gauge needles were inserted into the left medial thigh and the left gastrocnemius muscle. The electrode wires were then connected to the needles and plugged into the stimulator (Electronic Stimulator, Model SEN-7203, NIHON KOHDEN, Tokyo, Japan). The stimulating voltage was set at 5 V for 1 millisecond to cause noticeable contraction of the left hind limb. The stimulation frequency was 3 Hz, and the left hind limb was electrically stimulated for 30 minutes. Arterial and venous blood was sampled to measure the oxygen saturation before stimulation and at 15 and 30 minutes after stimulation.

A mouse model of hind limb ischemia and treatments. Male wild-type and Flt-1 tyrosine kinase deficient (Flt-1 TK$^{-/-}$) mice[24] were used. After anesthesia, unilateral hind limb ischemia was induced in the mice as previously described.[10,25] Additional details are provided in the Appendix (online only).

Statistical analyses. Data are expressed as mean ± standard error of the mean. Statistical analysis was assessed by one-way or two-way analysis of variance with post hoc test. Values of $P < .05$ were considered statistically significant.

RESULTS

Effects of statins and pitavastatin-NP on the angiogenic capacity of HECs in vitro. Treatment with pitavastatin increased angiogenic activity in HECs, whereas other statins had no effect (Fig 1). Treatment with pitavastatin-

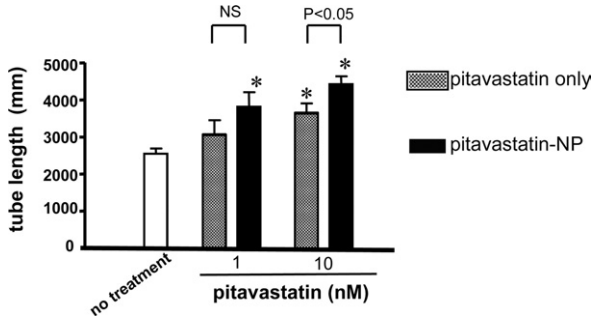

Fig 2. Effects of pitavastatin and pitavastatin nanoparticles *(NP)* are shown on the angiogenic capacity of human endothelial cells in vitro by quantitative analysis of tube formation (tube length per well) of six independent experiments. $*P < .01$ vs control by two-way analysis of variance with the Dunnett multiple comparison test.

NP increased angiogenic activity in HECs. The angiogenic activity of statin-NP was greater than that of 10 nM pitavastatin alone (Fig 2).

Effects of pitavastatin-NP on angiographically visible collateral arterial development. Because only a single dose of pitavastatin (0.4 mg/kg)-NP was previously examined in the mouse model,[13] the dose-response relationship of pitavastatin-NP with angiographically visible collateral arterial development (arteriogenesis) was examined in the present study. Treatment with pitavastatin (0.5 mg/kg)-NP, but not with those with pitavastatin at 0.05 or 0.15 mg/kg, increased the arteriogenic response, as assessed by the angiographic score (Fig 3, *A*). Representative angiograms 28 days after treatment demonstrate corkscrew-like collateral arterial development only in the pitavastatin-NP group (Fig 3, *B*). Treatment with pitavastatin (0.5 mg/kg)-NP significantly increased the angiographic score (Fig 3, *C*). In contrast, no treatment effects on the angiographic score were noted in the FITC-NP or pitavastatin-only groups.

Effects of pitavastatin-NP on histopathologic angiogenesis and arteriogenesis. Treatment with pitavastatin (0.5 mg/kg)-NP, but not with FITC-NP or statin only, significantly increased the capillary density and capillary/muscle fiber ratio, which are indices of angiogenesis (Fig 4, *A*). The beneficial effects of pitavastatin-NP were not associated with significant changes in serum biochemical markers (Table). Treatment with pitavastatin-NP also significantly increased the α-SMA$^+$ arteriolar density and arteriole/muscle fiber ratio, which are indices of arteriogenesis (Fig 4, *B*), indicating that pitavastatin-NP treatment induced angiogenesis and arteriogenesis.

Examination of hematoxylin-eosin-stained sections revealed no abnormal histopathologic findings (inflammation and fibrosis) among the four groups (data not shown). There was no significant difference in muscle fiber density among the four groups (PBS groups: 129 ± 8, 145 ± 4/mm^2; FITC-NP groups: 130 ± 3 and 129 ± 6/mm^2).

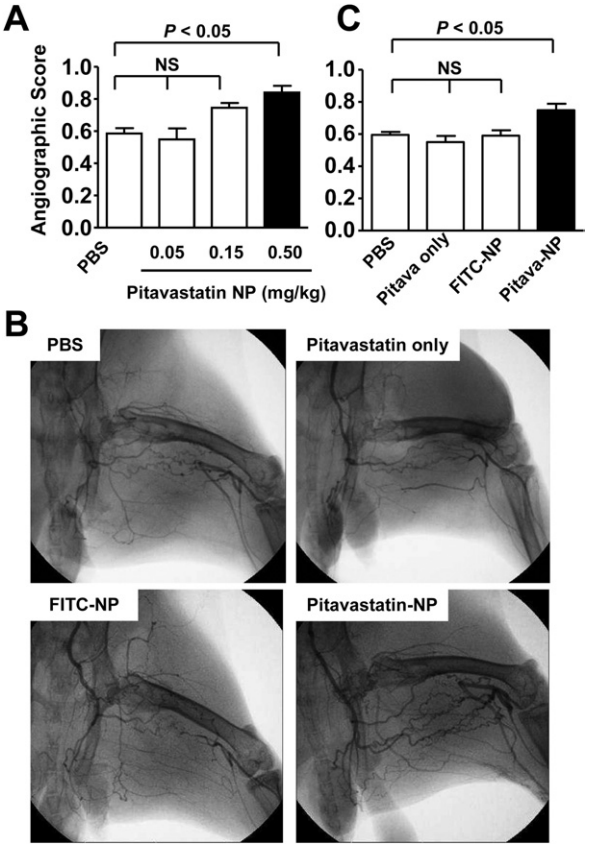

Fig 3. Effects of pitavastatin nanoparticles (*NP*) on angiographically visible collateral arterial development are shown 28 days after treatment. **A,** Effects of pitavastatin-NP containing 0.05, 0.15, or 0.5 mg/kg pitavastatin on the angiographic score (n = 3 each). **B,** Representative angiograms are shown of the phosphate buffered saline *(PBS)*, pitavastatin-only, fluorescein isothiocyanate *(FITC)*-NP, and pitavastatin-NP groups at 28 days after treatment. Corkscrew-like collateral arteries were observed only in the pitavastatin-NP group. **C,** Summary of the angiographic scores obtained for the four groups in panel **B** (n = 6 each).

Effects of pitavastatin-NP on tissue oxygen saturation. The tissue oxygen pressure in adductor skeletal muscles of the ischemic limb was measured 28 days after treatment. Treatment with pitavastatin (0.5 mg/kg)-NP significantly increased tissue oxygen pressure compared with the other groups (Appendix Fig II, online only).

Endothelial cell-selective delivery of NP. The cellular distribution of FITC was examined 3, 7, and 28 days after the intramuscular injection of FITC-NP or FITC only. On day 3 after injection, strong FITC signals were detected in FITC-NP–injected ischemic muscle (Fig 5, *A*), whereas no FITC signals were observed in control nonischemic muscle (Fig 5, *A*) or in ischemic muscle injected with FITC only (data not shown). The FITC signals were localized predominantly to the capillaries and arterioles. Weak FITC signals were also detected in myocytes at day 3t. On day 7 and 28, FITC signals remained localized predomi-

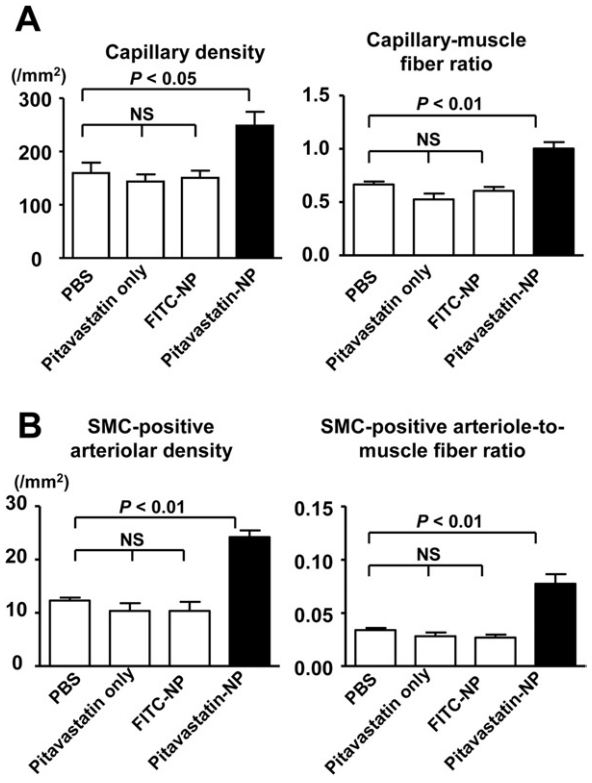

Fig 4. Effects of pitavastatin nanoparticles (*NP*) on angiogenesis and arteriogenesis are shown 28 days after treatment. **A,** CD-31$^+$ capillary density and capillary/muscle fiber ratio (indices of angiogenesis) is shown in ischemic muscles (n = 6 each). **B,** α-Smooth muscle actin (α-SMA)-positive arteriolar density and arteriole/muscle fiber ratio is shown in ischemic muscles (indices of arteriogenesis; n = 8 each). *FITC,* Fluorescein isothiocyanate; *PBS,* phosphate-buffered saline; *SMC,* smooth muscle cells.

nantly to capillaries and arterioles (Fig 5, *A*). Immunofluorescent staining revealed that FITC signals localized mainly to CD31$^+$ endothelial cells in FITC-NP–injected ischemic muscle 28 days after ischemia (Fig 5, *B*). In contrast, no FITC signals were observed in skeletal muscle myocytes on day 7 and 28 or in contralateral nonischemic hind limbs or remote organs (liver, spleen, kidney, and heart) at any time point (data not shown).

Effects of pitavastatin-NP on exercise-induced ischemia induced by electrical stimulation. To assess the functional efficacy of pitavastatin-NP on collateral arterial development, the effects of pitavastatin-NP on exercise-induced ischemia by electrical stimulation were examined. In the control PBS group, venous oxygen saturation in ischemic muscle decreased, and thus the difference in arteriovenous oxygen saturation increased after 15 and 30 minutes of electrical stimulation (Fig 6, *A*), suggesting the occurrence of exercise-induced ischemia. Treatment with pitavastatin-NP, but not with FITC-NP or pitavastatin only, abrogated the increase in arteriovenous oxygen difference in the ischemic limb (Fig 6, *B*). There were no significant differences in systemic blood hemoglobin levels among the four groups (data not shown).

Effects of vatalanib-NP on angiogenesis and arteriogenesis induced by pitavastatin-NP. We recently reported in a murine model that therapeutic neovascularization induced by pitavastatin-NP was mediated by increased eNOS activity and multiple endogenous angiogenic growth factors, such as VEGF.[10,25] Consequently, we examined VEGF expression in the four groups 28 days after treatment by immunohistochemistry and found increased VEGF positivity in CD31$^+$ endothelial cells of the capillaries and arterioles in the pitavastatin-NP group compared with other groups (Appendix Fig III, online only). Interestingly, positive VEGF staining was also detected in myocytes in the pitavastatin-NP group.

Vatalanib was selected because this molecule inhibits receptor tyrosine kinases of VEGR receptor types 1-3. Treatment with vatalanib-NP elicited no effects on angiographically visible collateral arterial development induced by hind limb ischemia in animals treated with PBS; however, it abrogated the arteriogenic response induced by pitavastatin-NP (Fig 7, *A* and *B*). In addition, treatment with vatalanib-NP abrogated histopathologic, angiogenic (capillary density), and arteriogenic (arteriolar density) responses induced by pitavastatin-NP (Fig 7, *C*). Vatalanib-NP elicited significant effects on histopathologic arteriogenic (arteriolar density) responses under baseline conditions (Fig 7, *C*).

Effects of pitavastatin-NP on angiogenesis and arteriogenesis in flt-1 TK$^{-/-}$ mice transfected with and without the *sFlt-1* gene. To examine the role of VEGF receptors (flk-1 and flt-1), the effects of pitavastatin-NP on ischemia-induced neovascularization were examined in wild-type and flt-1 TK$^{-/-}$ mice (Appendix Fig IV, online only). Compared with wild-type mice, the therapeutic effects of pitavastatin-NP decreased but were still observed in flt-1 TK$^{-/-}$ mice. To further examine the role of flk-1, *sFlt-1* gene transfer was performed into flt-1 TK$^{-/-}$ mice. The *sFlt-1* gene transfer blunted the therapeutic effects of pitavastatin-NP.

DISCUSSION

The present study demonstrates that NP-mediated endothelial cell-selective delivery of pitavastatin increased the development of collateral arteries (arteriogenesis) and improved exercise-induced ischemia in a rabbit model of chronic hind limb ischemia, indicating that this novel cell-selective delivery system is feasible for therapeutic arteriogenesis. We selected this rabbit model for translation to clinical settings in humans because it represents a preclinical model of arteriogenesis after femoral artery occlusion,[26] as observed in patients with severe peripheral artery disease.

Stimulation of the growth of collateral arteries (arteriogenesis) is evolving as a new therapeutic option for patients with atherosclerotic occlusive vascular disease, even though induction of additional angiogenesis or vasculogenesis is beneficial.[11,13] We assumed that the vascular endothelium would be an appropriate cellular target for the development

Table. Serum biochemical profiles

Variable[a]	*PBS*	*FITC-NP*	*Pitavastatin only*	*Pitavastatin-NP*
CPK (U/L)				
Day 7	345 ± 30	766 ± 270	445 ± 98	385 ± 44
Day 14	279 ± 8	486 ± 38	459 ± 118	296 ± 18
Day 21	242 ± 16	535 ± 58	396 ± 72	252 ± 12
Day 28	275 ± 60	229 ± 15	275 ± 33	259 ± 31
AST (IU/L)				
Day 7	10 ± 1	19 ± 3	16 ± 2	10 ± 1
Day 14	7 ± 0.3	19 ± 3	19 ± 6	8 ± 3
Day 21	15 ± 1	20 ± 2	22 ± 7	19 ± 4
Day 28	31 ± 11	29 ± 6	18 ± 2	20 ± 2
ALT (IU/L)				
Day 7	36 ± 9	36 ± 10	38 ± 5	29 ± 11
Day 14	26 ± 1	34 ± 6	37 ± 7	33 ± 9
Day 21	38 ± 7	33 ± 5	37 ± 8	43 ± 11
Day 28	42 ± 8	41 ± 10	35 ± 11	53 ± 19
BUN (mg/dl)				
Day 7	24 ± 0.2	17.3 ± 0.2	18 ± 1.5	24 ± 2
Day 14	23 ± 1	19.6 ± 2	24 ± 2	25 ± 1
Day 21	19 ± 1	18 ± 4	20 ± 2	19 ± 0.4
Day 28	26 ± 1	17 ± 0.3	17 ± 0.4	29 ± 2
Creatinine (mg/dL)				
Day 7	0.66 ± 0.01	0.82 ± 0.07	0.89 ± 0.01	0.80 ± 0.11
Day 14	0.70 ± 0.04	0.81 ± 0.09	0.87 ± 0.08	0.73 ± 0.05
Day 21	0.95 ± 0.02	0.80 ± 0.06	0.95 ± 0.01	1.03 ± 0.02
Day 28	0.85 ± 0.08	0.91 ± 0.06	0.86 ± 0.03	0.92 ± 0.03
Total cholesterol (mg/dL)				
Day 7	31 ± 10	31 ± 1	19 ± 5	46 ± 5
Day 14	26 ± 8	24 ± 3	19 ± 1	31 ± 4
Day 21	29 ± 10	18 ± 1	18 ± 3	32 ± 6
Day 28	18 ± 3	18 ± 2	21 ± 1	17 ± 2

ALT, Alanine aminotransferase; *AST,* aspartate transaminase; *BUN,* blood urea nitrogen; *CPK,* creatinine phosphokinase.
[a]Data are mean ± standard error of the mean (n = 3 each).

of collateral arteries after arterial occlusion because the endothelium plays a central role in the mechanism of arteriogenesis by expressing multiple growth factors and by recruiting monocytes and smooth muscle cells. We found that FITC signals were localized mainly to the vascular endothelium for up to 4 weeks after the injection of FITC-NP into ischemic skeletal muscles of rabbits in vivo, indicating that this NP-mediated delivery system may be useful as an innovative strategy for a therapy targeting endothelial cells. We recently reported that after cellular delivery of NP by endocytosis into endothelial cells, the PLGA NP escapes from the endosomal compartment to the cytoplasmic compartment and is retained in the cytoplasm, where release of the encapsulated drug occurs slowly in conjunction with the hydrolysis of PLGA.[10,27-29]

Daily administration of statins at high doses has been reported to augment arteriogenesis in normocholesterolemic rabbits.[6] These pleiotropic effects of statins are mediated through reduced levels of cholesterol biosynthesis pathway intermediates that serve as lipid attachments for post-translational modification (isoprenylation) of proteins, including Rho and Rac. Pitavastatin was selected as the NP compound because (1) pitavastatin elicited the most potent effects on the angiogenic activity of HECs in vitro compared with other statins, and (2) NP-mediated intracellular delivery of pitavastatin showed greater angiogenic activity of HECs compared with pitavastatin alone (Figs 1 and 2).

We also found in an in vivo rabbit model that (1) a single intramuscular injection of pitavastatin-NP increased the angiographic score in a dose-dependent manner, (2) pitavastatin (0.5 mg/kg) -NP significantly increased arteriogenesis and tissue oxygen pressure (tissue perfusion), and (3) the treatment of pitavastatin-NP increased immunoreactive VEGF expression selectively in vascular endothelial cells in the ischemic limb. Therefore, it is likely that that after NP-mediated endothelial delivery, pitavastatin is slowly released from the NP into the cytoplasm, resulting in significant therapeutic effects. Sata et al[8] reported that systemic daily administration of pitavastatin (1 mg/kg/day × 49 days = 49 mg/kg) has significant therapeutic effects in mice with hind limb ischemia. In our previous study, we reported the efficacy of pitavastatin (0.4 mg/kg)-NP in a murine model.[10] Therefore, at an approximately 100-fold lower dose, our NP-mediated delivery system is as effective as the cumulative systemic dose.

In clinical trials that examined the effects of a single vascular growth factor on peripheral and coronary artery disease, clinical end points such as increased exercise tolerance were negative or disappointing, although increased vascularity was noted.[14,15] It has been reported that limb hemodynamics, such as ankle-brachial index or muscle

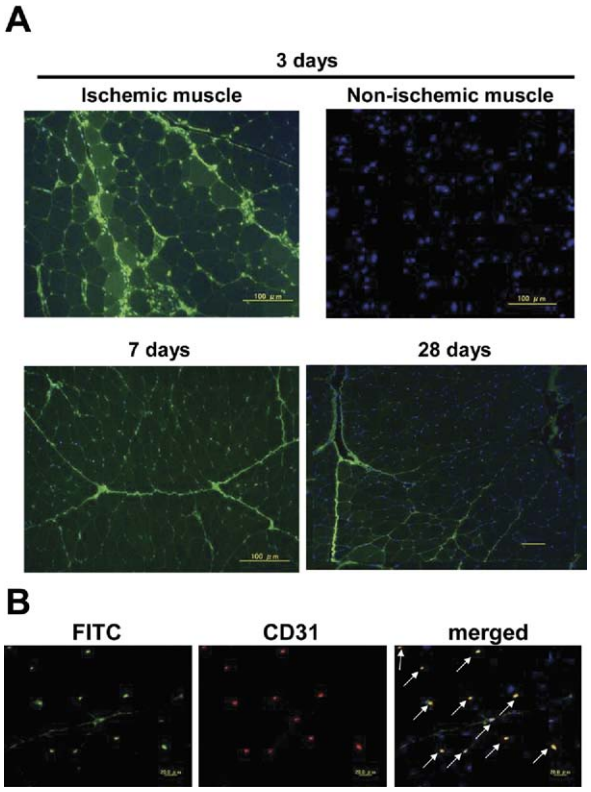

Fig 5. Cellular distribution of nanoparticles is shown in ischemic muscles. **A,** Fluorescent photomicrographs show cross sections of control nonischemic muscle and ischemic muscles at 3, 7, and 28 days after fluorescein isothiocyanate *(FITC)* nanoparticle *(NP)* injection. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole *(blue)*. Fluorescence microscopic settings (exposure, filter, excitation light intensity, etc.) were the same for all images. Scale bar = 100 μm. **B,** Photomicrographs of cross sections of ischemic muscle 28 days after FITC-NP injection stained immunohistochemically with the endothelial marker CD31 *(red)*. Most FITC signals colocalized with the vascular endothelium *(arrows)*. Scale bars = 20 μm.

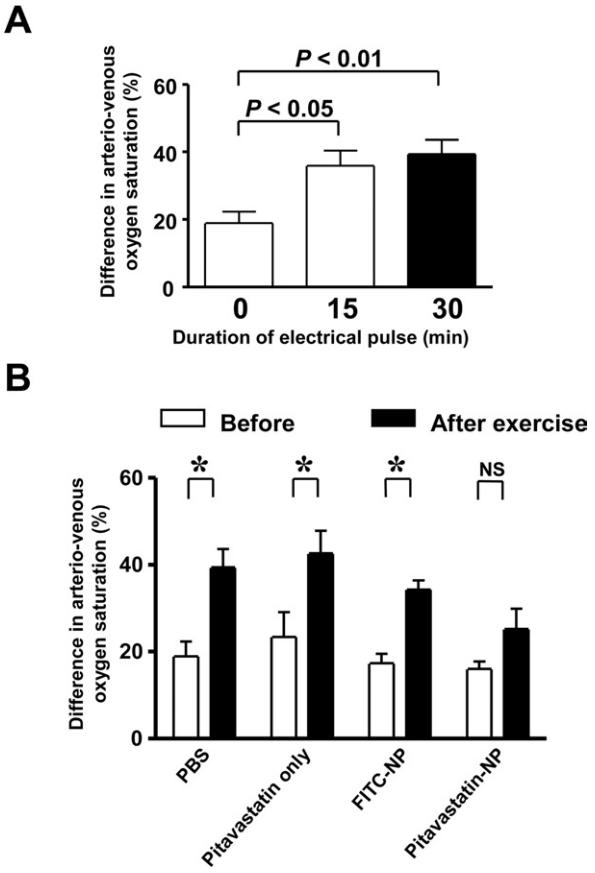

Fig 6. Effects are shown of pitavastatin nanoparticles *(NP)* on exercise-induced ischemia induced by electrical stimulation. **A,** Oxygen saturation in the femoral artery and vein in ischemic muscle is shown before and 15 and 30 minutes after muscular exercise by electrical stimulation in the phosphate-buffered saline (*PBS*) group (n = 6 each). **B,** The difference in arterial and venous oxygen saturation after 30 minutes of electrical pulse is shown in the four groups (n = 6 each). *FITC,* Fluorescein isothiocyanate.

blood flow at rest, are not correlated with functional capacity (claudication time or walking distance) in patients with peripheral arterial disease.[30] Therefore, assessment of the functional capacity of neovessels is needed in preclinical studies in animals. In other words, the improved functional capacity of collateral arteries must be a clinically important therapeutic goal in preclinical studies; however, few previous preclinical studies have addressed this point.

In the present study, we demonstrate that the arteriovenous oxygen difference in the ischemic hind limb increased in response to exercise in the PBS group, suggesting the development of exercised-induced ischemia. Treatment with pitavastatin-NP, but not with FITC-NP or pitavastatin only, prevented the development of exercise-induced ischemia. These data suggest that therapeutic arteriogenesis induced by pitavastatin-NP is associated with improved functional capacity.

We previously reported that the beneficial therapeutic effects induced by pitavastatin-NP are mediated by increased eNOS activity and multiple endogenous angiogenic growth factors in a murine model.[10] Recent reports by others have shown that mice lacking VEGF receptor 1 or placenta growth factor (a specific agonist of VEGR receptor 1), but not those lacking VEGF receptor 2, display impaired development of ischemia-induced angiogenesis and arteriogenesis.[31-33] However, roles of endogenous angiogenic growth factors in the mechanism of therapeutic effects of pitavastatin-NP have not been addressed.

In the present study, vatalanib-NP abrogated arteriogenic and angiogenic responses to pitavastatin-NP in rabbits. Furthermore, experiments with flt-1 $TK^{-/-}$ mice transfected with or without the *sFlt-1* gene showed partial contribution of both flt-1 and flk-1 to therapeutic angiogenic effects of pitavastatin-NP. These findings suggest that pitavastatin-NP produces an integrative system to form

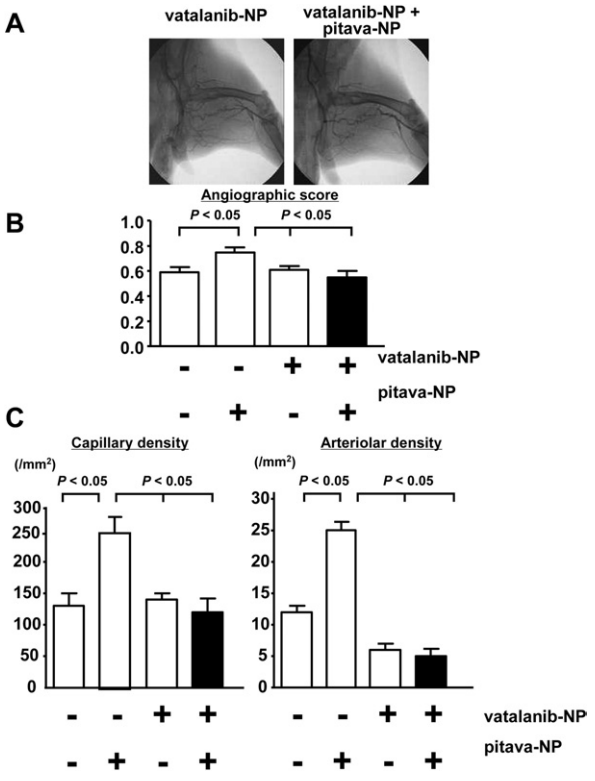

Fig 7. Effects of vatalanib nanoparticles *(NP)* are shown on angiogenesis and arteriogenesis induced by pitavastatin-NP. **A,** Representative angiograms show vatalanib-NP only and vatalanib-NP plus pitavastatin-NP groups 28 days after treatment. **B,** Summary of the angiographic scores obtained for the four groups (n = 3 each). **C,** Effects of vatalanib-NP are shown on histopathologic angiographic (capillary density) and arteriogenic (SMC-positive arteriolar density) responses induced by pitavastatin-NP.

functionally mature collaterals by controlled expression of endogenous VEGF and its receptor signals.

There are several limitations to the present study. First, only a single intramuscular injection of pitavastatin-NP was examined. In clinical settings, repetitive administration of an optimal dose may produce greater therapeutic effects. Second, we did not examine the contribution of bone marrow-derived progenitor cells because appropriate antibodies for detecting endothelial or smooth muscle progenitor cells are not available in rabbits. Further studies are needed to examine whether therapeutic effects afforded by pitavastatin-NP are associated with an increase in circulating endothelial progenitor cells.

CONCLUSIONS

This nanotechnology platform for vascular endothelial cell-selective delivery of pitavastatin is a promising strategy for the treatment of patients with severe organ ischemia and represents a significant advance in therapeutic arteriogenesis over current approaches. The nanotechnology platform may be further developed as a more effective and safer approach for therapeutic neovascularization.

AUTHOR CONTRIBUTIONS

Conception and design: SO, KE
Analysis and interpretation: SO, RN, KN, KE
Data collection: SO, RN, KN, KE
Writing the article: SO, KN, TM, KE
Critical revision of the article: SO, RN, KN, KE
Final approval of the article: SO, RN, KN, TM, MK, KS, RT, KE
Statistical analysis: SO, KN, KE
Obtained funding: KE
Overall responsibility: SO, KE

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Submitted Aug 19, 2009; accepted Mar 10, 2010.

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