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

Mitsuki Kubo, Kensuke Egashira, Takahiro Inoue, Jun-ichiro Koga, Shinichiro Oda, Ling Chen, Kaku Nakano, Tetsuya Matoba, Yoshiaki Kawashima, Kaori Haru, Hiroyuki Tsujimoto, Katsuo Sueishi, Ryuji Tominaga, Kenji Sunagawa

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 Vase Biol. 2009;29:00-00.)

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. These nonoptimal clinical results were in contrast to the results obtained in animal experiments and small open-label clinical trials. 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, 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, angioma-like capillary formation, and tumor-angiogenesis. 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 polyactide/glycolide copolymer (PLGA). The PLGA NP offers the advantages of safety, delivery of encapsulated drugs into the cellular cytoplasm, and slow cytoplasmic drug release. PLGA NPs are effectively and rapidly taken up by vascular endothelial cells in vitro. 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. Statins increase the angiogenic activity of mature endothelial cells as well as that of endothelial progenitor cells (EPCs) and augment collateral growth in ischemic heart and limb in experimental animals. In addition, statins attenuate atherosclerosis formation and have little potential risk of tumor angiogenesis in contrast to angiogenic growth factors. Most of these beneficial effects of statin on therapeutic neovascularization, however, were observed after daily administration of high doses, which...
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.

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.

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 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).
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 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 endocytosis, 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. 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.\(^2\) reported that systemic daily administration of pitavastatin (1 mg/kg per day \(\times\) 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.\(^2\) by showing that oral daily administration of pitavastatin for 14 days (1 and 10 mg/kg per day \(\times\) 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 IV) 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.

**Acknowledgments**

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

**Sources of Funding**

This study was supported by Grants-in-Aid for Scientific Research (19390216, 19650134) from the Ministry of Education, Science, and Culture, Tokyo, Japan, and by Health Science Research Grants (Research on Translational Research and Nanomedicine) from the Ministry of Health, Labor, and Welfare, Tokyo, Japan.

**Disclosures**

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

**References**


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Arterioscler Thromb Vasc Biol. published online March 26, 2009;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplement Data

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Department of Cardiovascular Medicine (MK, KE, TI, JK, LC, KN, TK and K Sunagawa), Surgery (SO, RT), and Pathology (K Sueishi), Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan, School of Pharmaceutical Science (YK), Aichi Gakuin University, Aichi, Japan, and Hosokawa Powder Technology Research Institute (KH, HT), Osaka, Japan.
**Supplementary Table 1.**
Serum biochemical profiles 3, 7 and 14 days after hindlimb ischemia

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<tr>
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<td>74±3</td>
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Data are mean±SEM (n=3 each)
Supplementary Table 2.
Tissue and serum pitavastatin concentrations after intramuscular injection of statin

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<td>Pitavastatin at 0.4 mg/kg</td>
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<td>muscle (ng/g tissue)</td>
<td>305 ± 80</td>
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<tr>
<td>serum (ng/ml)</td>
<td>3 ± 0.3</td>
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<tr>
<td>Statin-NP containing 0.4 mg/kg of pitavastatin</td>
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<tr>
<td>muscle (ng/g tissue)</td>
<td>2088 ± 412*</td>
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<tr>
<td>serum (ng/ml)</td>
<td>5 ± 0.7</td>
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</table>

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 immunostaining 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.

A

HUVEC  SkMC

% of FITC-positive cells

HUVEC  SkMC

P<0.01

B

Cellular fluorescence intensity (arbitrary units)

HUVEC  SkMC

P<0.001  P<0.001  NS  NS

FITC-NP (mg/ml)  0  0.1  0.5  0.1  0.5

CPZ (μM)  0  0  0  10  10

C

1 hour  3 days  7 days
**Supplementary Figure III Kubo M et al.**

**A**

Day 7

- No treatment
- Pitavastatin (mg/kg): 4, 20

Day 14

- No treatment
- Pitavastatin (mg/kg): 4, 20

**B**

Non-ischemic control  Ischemia – no treatment  Ischemia – statin-NP

**C**

% of Sca-1+/Flk-1+ cells

- Non-ischemic control
- No treatment
- Statin-NP

7 days after ischemia  14 days after ischemia
Supplementary Figure IV Kubo M et al.

The graph shows the ischemia/normal blood flow ratio with oral administration of Pitavastatin (mg/kg) and statin-NP (0.4 mg/kg). The results indicate a significant increase in blood flow with increasing doses of Pitavastatin and statin-NP compared to the no treatment condition.
Supplementary Figure VI Kubo M et al.

<table>
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Supplementary Figure VII Kubo M et al.

A

no treatment
3 days

no treatment
7 days

no treatment
14 days

statin-NP
3 days

statin-NP
7 days

statin-NP
14 days

VEGF
CD31
merged
Supplementary Figure VII Kubo M et al.

B

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</table>
A Co-treatment protocol

B Pre-treatment protocol
Supplement Material

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

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Department of Cardiovascular Medicine (MK, KE, TI, JK, LC, KN, TK and K Sunagawa), Surgery (SO, RT), and Pathology (K Sueishi), Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan, School of Pharmaceutical Science (YK), Aichi Gakuin University, Aichi, Japan, and Hosokawa Powder Technology Research Institute (KH, HT), Osaka, Japan.
Materials and Methods

Preparation of PLGA NP

A lactide/glycolide copolymer (PLGA) with an average molecular weight of 20,000 and a copolymer ratio of lactide to glycolide of 75:25 (Wako Pure Chemical Industries, Osaka, Japan) was used as a wall material for the NP. According to manufacturer’s instruction, a bioabsorption half-life of this product is 2 weeks in rat tissue. Polyvinylalcohol (PVA-403; Kuraray, Osaka, Japan) was used as a dispersing agent. Fluorescein-isothiocyanate (FITC; Dojin Chemical, Tokyo, Japan) was used as a fluorescent marker of the NP.

PLGA NP incorporated with FITC or pitavastatin (Kowa Pharmaceutical Co. Ltd., Tokyo, Japan) were prepared by a previously reported emulsion solvent diffusion method in purified water. PLGA were dissolved in a mixture of acetone and methanol. Then, FITC or pitavastatin were added into this solution. The resultant polymer-FITC or polymer-statin solution was emulsified in PVA solution under stirring at 400 rpm using the propeller-type agitator with three blades (Heidon 600G; Shinto Scientific, Japan). After agitating the system for 2 h under reduced pressure at 40 °C, the entire suspension was centrifuged (20,000×g for 20 min at -20 °C). After removing the supernatant, purified water was added to mix with the sediment. The wet mixture was then centrifuged again to remove the excess PVA and the unencapsulated reagent that could not adsorb on the surfaces of NP. After repeating this process, the resultant dispersion was freeze-dried under the same conditions. The FITC- and pitavastatin-loaded PLGA NP contained 5 % (w/v) FITC and 5 % (w/v) pitavastatin, respectively.

Particle size and surface charge measurements

Scanning electron microscopy picture of the PLGA NP indicates that the NP is prepared in the form of powder. The mean particle size was analyzed by light scattering.
method (Microtrack UPA150; Nikkiso, Tokyo, Japan). A sample of nanoparticulate suspension in distilled water was used for particle size analysis. The diameter of PLGA NP was 196 ± 29 nm. Surface charge (zeta potential) was also analyzed by Zetasizer Nano (Sysmex, Hyogo, Japan) and was anionic charge (-15±10 mV at pH 4.4).

Bioabsorption process of PLGA NP
The chemical structure of PLGA and its bioabsorption process (hydrolysis) are indicated below. Scanning electron microscopic examination of time course of biodegradation in phosphate buffer solution shows slow degradation of NP with time.

Bioabsorption Process (hydrolysis) of PLGA
Intracellular uptake and intracellular distribution of NPs

Human umbilical vein endothelial cells (HUVEC) were obtained from Cambrex BioScience Walkersville, Inc., cultured in EGM-2 (Lonza, Charles City, IA) with supplements (Lonza), and used between passages 4 to 8. Human skeletal muscle cells (SkMC) were also obtained from Cambrex BioScience Walkersville, Inc. and cultured in SkGM (Lonza).

The HUVEC and SkMC were seeded on the 8-well-chamber slide to an initial concentration of 1.5 × 10⁴ cells per well and incubated at 37 °C/5 % CO₂ environment until cells were subconfluent. The growth medium was replaced with the FITC-NP suspension medium (0.1 to 0.5 mg/ml) without supplements and then further incubated for 1 hour. The cells were then washed three times with PBS to eliminate extracellular NP as previously described. Then, the cells were fixed with methyl-alcohol and nuclei were counterstained with propidium iodide (PI; vector shield). Intracellular uptake of FITC-NP was evaluated by fluorescence microscopy (Biozero; KEYENCE, Osaka, Japan). The number of cells in 5 random fields was manually counted and cellular uptake percentage was assessed by the percentage of fluorescence positive cells per total cells.

In another set of experiments using HUVEC, the growth medium was replaced with FITC-NP (0.5 mg/ml) and further incubated for 1 hour. After excess extracellular NP were washed with PBS, the cells were cultured with normal growth medium and intracellular retention of FITC was examined as described above at days 3 and 7.

To investigate the potential mechanism of cellular uptake of NP, HUVEC and SkMC were seeded on the 96-well plate in the presence or absence of an inhibitor of clathrin-mediated endocytosis pathway, chlorpromazine (CPZ; Sigma) at 10 or 30 μM for 30 min at 37 °C in the culture medium without supplements, and then incubated with FITC-NP suspension medium (0.1 to 0.5 mg/ml) for further 30 min. After incubation, cells were washed and lysed with triton X and NaOH and then the amount of fluorescence in each well were analyzed with fluorescence-plate reader (Mithras LB940; BERTHOLD BIOTECHNOLOGY, Germany).

Angiogenesis Assay of Human Endothelial Cell

Angiogenesis assay of human endothelial cells was tested by 2-dimensional Matrigel assay as previously described. HUVECs (2×10⁴) were suspended on the 8-well-chamber slide pre-coated with 200 μl Matrigel (BD Bioscience) in 500 μl EBM-2 medium with supplements (Lonza) in the presence or absence of pitavastatin or NP incorporated with pitavastatin at 1 and 10 nM. In another set of experiments, HUVEC were pre-treated with pitavastatin or NP incorporated with pitavastatin at 1 and 10 nM for 24 hours and washed, and then the cells were suspended on the Matrigel.

After 24 hours of incubation on the Matrigel at 37 °C/5 % CO₂ environment, tube formation were quantified by light microscopy (Biozero; KEYENCE, Osaka, Japan) and the length of completed tube-like structures in 5 random fields was quantified in a blinded fashion in each experiment.

Animal Preparation and Experimental Protocol

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

Male 8-weeks-old C57BL/6J wild-type mice (Japan-Clea, Tokyo, Japan) were bred and maintained in the Laboratory of Animal Experiments at Kyushu University. After anesthesia with an intraperitoneal injection of ketamine hydrochloride (70 mg/kg) and
xyladine hydrochloride (3 mg/kg), we induced unilateral hindlimb ischemia to mice as previously described. Briefly, the proximal portion of the left femoral artery and vein including the superficial and deep branch as well as the distal portion of saphenous artery and vein were ligated and resected after all side branches were dissected free. Immediately after induction of ischemia, animals were randomly divided into 4 groups; a control no treatment group and others received intramuscular injections of FITC-NP (PLGA at 0.18 mg/100 µl) (NP group), intramuscular injections of pitavastatin at 0.01 mg/100 µl (0.4 mg/kg)(Pitava only group), or intramuscular injections of pitavastatin-NP [PLGA at 0.18 mg/100 µl containing 0.01 mg (0.4 mg/kg) of pitavastatin] (Pitava-NP group) into the left femoral and thigh muscles with a 27-gauge needle. This dose of pitavastatin NP was selected because we examined effects of pitavastatin-NP containing pitavastatin at 0.1, 0.4, 1.0 and 1.5 mg/kg In preliminary studies and confirmed that pitavastatin NP containing 0.4 mg/kg pitavastatin was an optimal dose in our experiments. Biochemical parameters listed in supplemental Table 1 were measured 3, 7, and 14 days after treatment.

In another set of experiments, effects of intramuscular injections of pitavastatin-NP were examined in eNOS−/− mice and wild-type mice chronically treated with Nω-nitro-L-arginine methyl ester (L-NAME; Sigma), an NO synthase inhibitor, in drinking water (2 mg/kg) from 7 days before operation to sacrifice. Two other groups received intramuscular injections of non-nanoparticulated soluble pitavastatin at high doses at 4 and 20 mg/kg. Furthermore, three other groups received systemic daily oral administration of pitavastatin at doses of 0.4, 1.0 and 10 mg/kg, solved in 0.5% carboxymethyl cellulose by gavage from the day of surgery until the mice were sacrifice on day 14.

**Histological and immunohistochemical analyses**

Histological evaluation was performed in 5-µm paraffin embedded sections from gastrocnemius muscle 14 days after hindlimb ischemia. Capillary and arteriolar density in ischemic muscle were determined by immunohistochemical staining with anti-mouse platelet endothelial cell adhesion molecule (PECAM)-1 antibody (CD31; Santa Cruz Biotechnology) and α-smooth muscle actin (α-SMA; DAKO), respectively. Digital images of 5 microscopic fields from 4 different sections from each animal were stored. Capillary density was expressed as the number of CD31 positive cells per mm² and arteriolar density was expressed as the number of circumvented brown signals of α-SMA per mm² as previously described. To determine intracellular molecular signals for angiogenesis, cross sections were stained with anti-phosphorylated-Akt antibody (Cell Signaling) or anti-phosphorylated-eNOS antibody (Cell Signaling). To determine cellular localization of angiogenic growth factors 3, 7 and 14 days after ischemia, cross sections were stained with anti-VEGF or anti-FGF-2 antibody with anti-PECAM-1 (CD31) antibody, as a primary antibody (all from Santa Cruz Biotechnology), and anti-mouse IgG (Alexa 488; Molecular Probes) or anti-rabbit IgG (FITC; Santa Cruz Biotechnology) with anti-goat IgG antibody (Alexa 555; Molecular Probes), as a secondary antibody, respectively. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI; Vector Shield).

**Distribution of nanoparticles in vivo**

Three, 7 and 14 days after hindlimb ischemia and intramuscular injection of FITC-NP, gastrocnemius muscle was isolated from ischemic and non-ischemic limbs, and FITC signals were examined under a fluorescent stereomicroscope. Frozen cross sections of those muscles were then prepared and examined under a fluorescent microscope (Biozero, KEYENCE, Osaka, Japan). Nuclei were counterstained with propidium iodide (PI; Vector Shield). Another sections were stained with anti-mouse PECAM-1 antibody (CD31; Santa
Cruz Biotechnology), as a primary anti-body, and anti-goat IgG (Alexa 555; Molecular Probes), as a secondary anti-body. Frozen cross sections of liver, spleen and kidney were also examined.

**Western blotting**

Homogenates of muscle tissues were analyzed for immunoblotting 7 days after induction of hindlimb ischemia. Proteins were separated in 7.5 % or 15 % SDS-polyacrylamide gels and then blotted onto a membrane. Membrane was incubated with antibodies against phosphorylated-Akt, phosphorylated-eNOS, Akt (1:1000, Cell Signaling), eNOS (1:1000, Affinity BioReagents), VEGF, FGF-2 and MCP-1 (1:200, Santa Cruz Biotechnology) and then the blots were reprobed with GAPDH (1:1000, Santa Cruz Biotechnology).

**Flow Cytometric Analyses of EPC Mobilization**

Peripheral blood was obtained from mice 7 and 14 days after hindlimb ischemia. EPC are thought to derive from mononuclear leukocytes that are positive for both Sca-1 and Flk-1 (vascular endothelial cell growth factor receptor-2)\(^1\)\(^2\). The percentage of mononuclear cells that were positive for both the Sca-1-FITC and Flk-1-PE antibodies (Pharmingen) was then analyzed with a FACS Caliber flow cytometer (Becton Dickinson).

**Measurements of statin concentration in serum and muscle tissue**

Statin concentration in serum and muscle were measured at predetermined time points by using column-switching high performance liquid chromatography (HPLC) system as previously reported\(^3\). Briefly, the column-switching HPLC system consists of two LC-10AD pumps, an SIL-10A auto-sampler, a CTO-10A column oven, a six-port column-switching valve and an SPD-10A UV-detector (all from Shimadzu, Kyoto, Japan). The column temperature was maintained at 40 °C. Preprepared serum or tissue homozynates sample solutions were injected from auto-sampler into HPLC system and the detection of statin in sample solutions was carried out at 250 nm with a UV-detector. The detected peak-area was measured with Lcsolution software (Shimadzu, Kyoto, Japan).

**References**

6. Weis M, Heeschen C, Glassford AJ, Cooke JP. Statins have biphasic effects on


