J Tokyo Wom Med Univ
77 (6) 306~314 (2007)

Effects of Hydrophilic Statin, Pravastatin, on Vasculogenic Properties of Endothelial Progenitor Cells

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(Accepted Mar. 26, 2007)

Previous studies have demonstrated that hydrophobic hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) mobilize circulating endothelial progenitor cells (EPCs), and contribute to therapeutic vasculogenesis. However, the effects of hydrophilic statin (pravastatin) on EPC kinetics remain to be investigated. Here, we investigated whether pravastatin stimulates EPC kinetics via intracellular signal transduction following its uptake by EPCs.

Pravastatin uptake by cultured EPCs was measured by ^{14}C-radio labeled molecules, and was compared with that by HepG2 cells as a negative control (*29.15 ± 2.80 vs 7.82 ± 0.47 count/min/mg protein, *$p<0.0001$). EPC migratory activity toward pravastatin was significantly stimulated when compared with the vehicle group. EPC antiapoptotic assay by DAPI staining and cell death detection ELISA demonstrated abrogated apoptosis in the pravastatin group. In addition, pravastatin activated the PI-3-kinase/Akt pathway leading to endothelial nitric-oxide synthase (eNOS) activation. Co-treatment with PI-3/Akt inhibitors blocked pravastatin-induced Akt activation, indicating Akt activation through PI-3-kinase phosphorylation. When pravastatin was orally administered to nude mice, the number of circulating EPCs increased in a time-dependent fashion.

These findings suggest that hydrophilic pravastatin exerts provasculogenic effects via the upregualtion of EPC migration and survival through PI-3-kinase/Akt/eNOS pathway activation following intracellular uptake.

Key words: EPC, pravastatin, vasculogenesis, angiogenesis, eNOS

Introduction

Hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase inhibitors, statins, have been developed as lipid-lowering agents and have been found to reduce morbidity and mortality from coronary artery disease[1)~3)]. Primary and secondary prevention trials recently suggested that statins also have pleiotropic effects, including cholesterol reduction[4)~6)]. Moreover, various investigations have revealed direct physiological or molecular actions in endothelial cells[7)~9)]. Kureishi et al reported that hydrophobic statins rapidly activate protein kinase Akt/PKB in endothelial cells, following phosphorylation of the endogenous Akt substrate eNOS[10)]. In addition, these compounds stimulate postnatal vasculogenesis via endothelial progenitor cells (EPCs) derived from bone marrow, which are known to incorporate into foci for physiological or pathological neovascularization[11)~15)].

Pravastatin is recognized as a "unique statin", due to its hydrophilic features that prevent penetration through the cell membrane. Pravastatin has been shown to inhibit cholesterol synthesis in vivo, specifically in the liver[16)], which is the major site of cholesterol synthesis. The fact that this inhibitory effect is restricted to the liver tissue has long been of

great pharmacological and toxicological interest. Considering the pharmacological kinetics of pravastatin and its various functions, despite its hydrophilicity, about 30% is absorbed after oral administration in healthy subjects and the oral bioavailability is about 20%, thus suggesting first-pass metabolism[17]. A human organic anion transporter, OATP2 (SLC21A6)[18][19], recently detected by Abe et al and Hsiang et al is expressed exclusively in intact hepatocytes, and was found to transported pravastatin to the intercellular space, but the transporter was not expressed in Hep G2 cells of a human hepatoma cell line, which do not transport pravastatin[19][20].

Another transporter of the OATP family, OATP-B (SLC21A9), is broadly expressed in several tissues[21][22], and was also found to transport pravastatin[23]. However, at present, the cellular features of such hydrophilic pravastatin uptake in cells other than hepatocytes remain to be investigated. Hence, we focused on the predictable provasculogeneic effects of pravastatin on EPC kinetics.

Materials and Methods

Materials

Pravastatin and radiolabeled [^{14}C] pravastatin sodium salt (specific activity: 14.36 μCi/μmol)were kindly supplied by Sankyo Co. Ltd. (Tokyo, Japan). Hep G2 cells were purchased from Japan Health Sciences Foundation (Tokyo, Japan). Experiments were initiated by addition of the indicated amount of pravastatin, 100 ng/ml vascular endothelial growth factor (VEGF; Peprotech EC, Ltd.) or vehicle control. All other chemicals used were of reagent grade.

Animals

All aspects of this study were approved by the Tokai University Animal Care and Use Committee. Female FVB/NJcl mice (n=6, 8-10 weeks old, 18 to 23 g weight, Clea Japan Co. Ltd., Tokyo, Japan) were fed with a daily oral dose of pravastatin (0.2 or 1 mg/kg/day) or vehicle (sterilized water) for two weeks and anesthetized with 160 mg/kg intraperitoneal pentobarbital for surgical procedure.

Human EPC Culture

Peripheral blood mononuclear cells (PBMNC) were isolated from the blood of human volunteers by density gradient centrifugation with Histopaque-1077 (Sigma, St. Louis, Missouri, USA), and were cultured for 7 days on human fibronectin-coated tissue culture dishes, as described previously[24)~26)]. Cultured EPCs were used for all experiments.

Uptake Experiments

[^{14}C] Pravastatin uptake by cultured EPCs was assessed as described previously[20]. Briefly, Hep G2 cells were cultured in DMEM (Sigma) with 10% fetal bovine serum and penicillin-streptomycin (100 U/100 mg/ml). Cultured human EPCs or Hep G2 cells at the cell density of 2×10^5/ml per well were reseeded onto 24-well tissue culture plates for 24 hr before the uptake study. To measure cellular uptake of pravastatin, medium was removed, cells were then washed with prewarmed, serum-free medium, and medium containing radiolabeled [^{14}C] pravastatin was added. Plates were incubated in a CO_2 incubator for 1 hr, and cells were washed with ice-cold phosphate-buffered saline and lysed with 0.1 N NaOH. A fraction of the lysate was used to determine radiolabel incorporation by liquid scintillation counting on a Packard 2550 TR/AB (PerkinElmer, Inc., Boston, Massachusetts, USA), and another fraction was used to determine protein concentration by Bradford assay with bovine serum albumin (BSA; Sigma) as a standard.

Migration assay and apoptosis assay

EPC migration was evaluated using a modified Boyden chamber assay, as described previously[24)25)]. Briefly, 1 μM pravastatin, 50 ng/ml VEGF or vehicle, in serum-free EBM-2 media with 0.1% BSA, was placed in the lower compartment of the chambers. A total of 1×10^5 EPCs in 100 μl of EBM-2 supplemented with 0.1% BSA were seeded in the upper compartment of the chambers. Cell migration was quantified by counting cells in 13 randomly selected high-power fields[24)25)]. All groups were studied in triplicate.

The antiapoptotic effects of pravastatin were assessed by cell death detection ELISA^{Plus} (Roche Diagnostics GmbH, Mannheim, Germany). Cultured EPCs were reseeded on 96-well plate two days before apoptosis assay. Media were replaced with serum-free EBM-2 media containing 1 μM pravasta-

tin, 50 ng/ml VEGF or vehicle at 12 hr before the assay. Plates were then centrifuged and supernatant containing necrotic DNA that leaked through the membrane during incubation was discarded. Pellets were lysed with lysis buffer, and an aliquot of the resulting mixture was transferred the ELISA plate. Immunolabeled plates were spectrophotometrically measured using a plate reader (Molecular Devices spectra MAX 250, Global Medical Instrumentation, Inc., Ramsey, Minnesota, USA) at 405 nm. Antiapoptotic activity was independently evaluated per 1×10^5 cells seeded on a 4-chamber slide and exposed to 1 μM pravastatin or vehicle for 24 hr. Pyknotic nuclei were manually counted after DAPI (Vector Laboratories, Inc., Burlingame, California, USA) staining, as described previously[27)28)]. All studies were performed in duplicate.

Western blot analysis

Cultured EPCs were stimulated for 5 min with or without pravastatin (10 μM). Afterwards, proteins were incubated in RIPA buffer for Western blot analysis. Phosphatase inhibitors were added to protein lysis buffer according to standard protocols. PI-3-kinase inhibitors, LY294002 (10 μM, Sigma) and wortmannin (100 nM, Sigma), were added 30 min prior to statin treatment. SDS-PAGE/Western blot was performed as described previously[29)]. Briefly, 7.5% polyacrylamide gels were used for electrophoresis. Proteins were transferred to PVDF membranes (Amersham). Membranes were incubated overnight with primary antibodies against phospho-Akt serine 473 (1 : 1,000; Cell Signaling), total Akt (1 : 1,000; Cell Signaling), phospho-eNOS serine 1,177 (1 : 1,000; Cell Signaling), total eNOS (1 : 1,000; Santa Cruz Biotechnology) followed by anti-rabbit secondary antibodies (1 : 2,000; Amersham Bioscience) for 2 hours. Bands were detected by enhanced chemiluminescence (CoolSaver AE-6955, ATTO; Tokyo).

RT-PCR

Total RNA from cultured EPCs was extracted using the SV total RNA isolation system (Promega Co., Madison, Wisconsin, USA). cDNA synthesis was performed with 0.75 μg of total RNA using the Advantage RT-for-PCR kit (Clontech, Palo Alto, California, USA). Reverse transcription-polymerase chain reaction (RT-PCR) products were analyzed by 2% agarose gel electrophoresis. The following oligonucleotides were used as forward and reverse primers to amplify the indicated cDNA: OATP2, 5′-CATGCTGATTGTTAAAATTGTTCAACC-3′ (forward), 5′-CCCTTAACAATGTGTTTCACTATCT-GC3′ (reverse).

Mouse EPC culture assay

PBMNCs were obtained by density gradient centrifugation with Histopaque-1083 (Sigma) from the blood of athymic nude mice orally treated with or without pravastatin at physiological doses (control, 0.2 mg/kg, or 1 mg/kg; n=5, 5, or 6) for 2 weeks. One week after culture, EPCs were assayed by uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated LDL (acLDL-DiI; Biomedical Technologies) and staining with FITC-conjugated BS-1 lectin (Vector Laboratories) to confirm endothelial lineage. Blinded investigators counted the stained EPCs by fluorescence microscopy[24)25)30)].

Statistical Analysis

All data are presented as means ± SEM. Differences between group means were assessed by unpaired Student's t test for single comparisons and by ANOVA for multiple comparisons. Statistical significance was assumed if the null hypothesis could be rejected at $p<0.05$.

Results

Cellular uptake of [^{14}C] Pravastatin by EPCs

HepG2 cells served as negative controls, because the cell line does not take up pravastatin due to the lack of OATP2[20)]. Uptake of pravastatin into cultured EPCs increased significantly in a dose-dependent manner as compared with Hep G2 cells (EPCs vs Hep G2: 10 μM pravastatin; *3.03 ± 0.24 vs 0.82 ± 0.07, 25 μM; *7.58 ± 1.22 vs 1.73 ± 0.07, 50 μM; *8.91 ± 0.83 vs 3.47 ± 0.90, 150 μM; *29.15 ± 2.80 vs 7.82 ± 0.47 count/min/mg protein, *$p<0.0001$, Fig. 1). Nevertheless, expression of OATP2 was not detected in EPCs by RT-PCR (data not shown). These findings suggest another mechanism for pravastatin uptake in EPCs.

Migratory and antiapoptotic effects of pravastatin in EPCs

The effects of pravastatin on EPC migration were analyzed using a modified Boyden chamber assay. The migratory activity of EPCs was significantly stimulated by 1 μM pravastatin (migrated cells / high power field; pravastatin vs vehicle: *29.7 ± 2.8 vs 19.7 ± 2.1, *p<0.01, Fig. 2) to the same extent as VEGF (Pravastatin vs VEGF: 29.7 ± 2.8 vs 29.8 ± 4.4, NS). Moreover, pravastatin showed antiapoptotic effects in serum starved EPCs, as confirmed by manually counting the apoptotic cells (% pyknotic nuclei change in EPCs; pravastatin vs vehicle: *56.2±4.4% vs 79.7 ± 4.1%, *p<0.01, Fig. 3A). The antiapoptotic effects of pravastatin were also verified by ELISA (pravastatin and VEGF vs vehicle: *0.66 ± 0.07 and *0.39 ± 0.04 vs 1.01 ± 0.08, *p< 0.0001, Fig. 3B).

On the other hand, pravastatin did not affect the proliferation of EPCs using the previously validated colorimetric MTS assay, as reported previously[25].

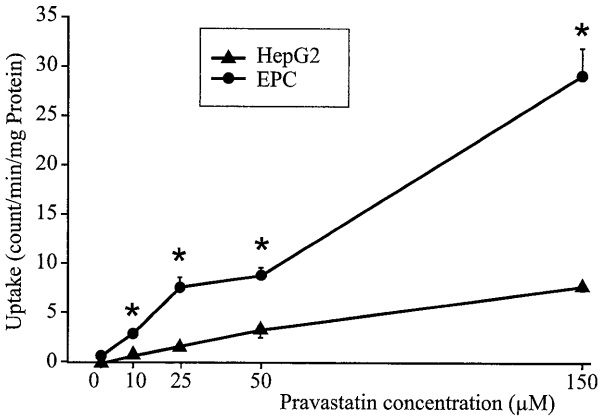

Fig. 1 Dose dependence of cellular uptake of pravastatin by EPCs (●) and HepG2 cells (▲)
* 29.15 + / − 2.80 vs 7.82 + / − 0.47 count/min/mg protein, *p < 0.0001.

Western blot analysis

In order to confirm whether the PI-3-kinase/Akt signaling pathway in EPCs activated by pravastatin, Western immunoblot analysis of Akt phosphorylation was performed with or without pravastatin. This assay revealed that pravastatin treatment at a concentration of 1 μM led to serine 473 Akt phosphorylation within 5 min (Fig. 4A). This Akt activation was abrogated by PI-3-kinase inhibitors, LY294002 (10 μM) and/or wortmannin (100 nM), suggesting that the PI-3-kinase/Akt signaling pathway is activated by pravastatin (Fig. 4A). Further-

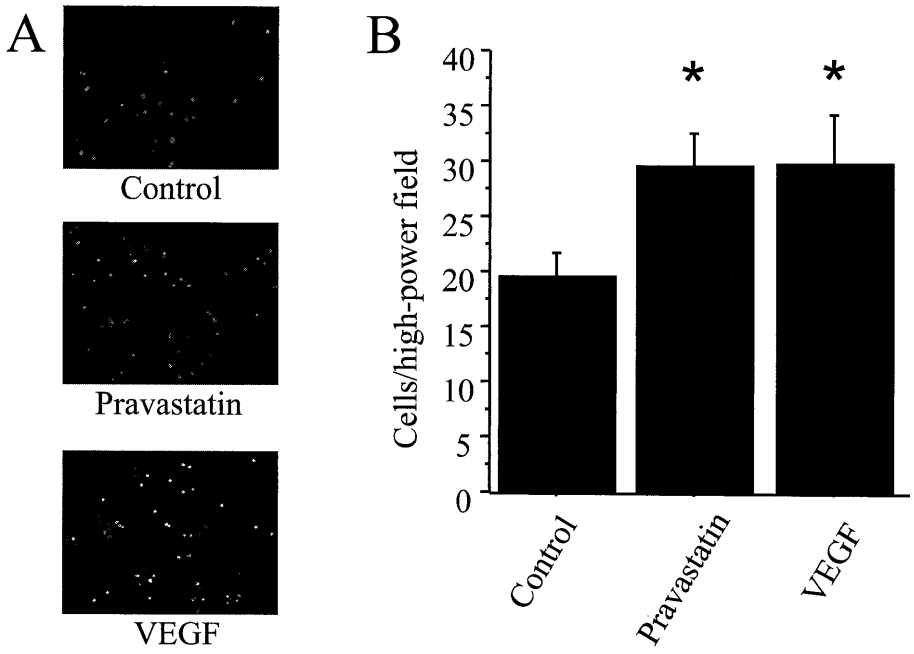

Fig. 2 Representative photomicrographs from each group of EPCs (A) and migratory response of EPCs toward the indicated dosage of pravastatin, as measured by modified Boyden chamber migration assay (B)
Migration activity was almost equivalent to that induced by VEGF.

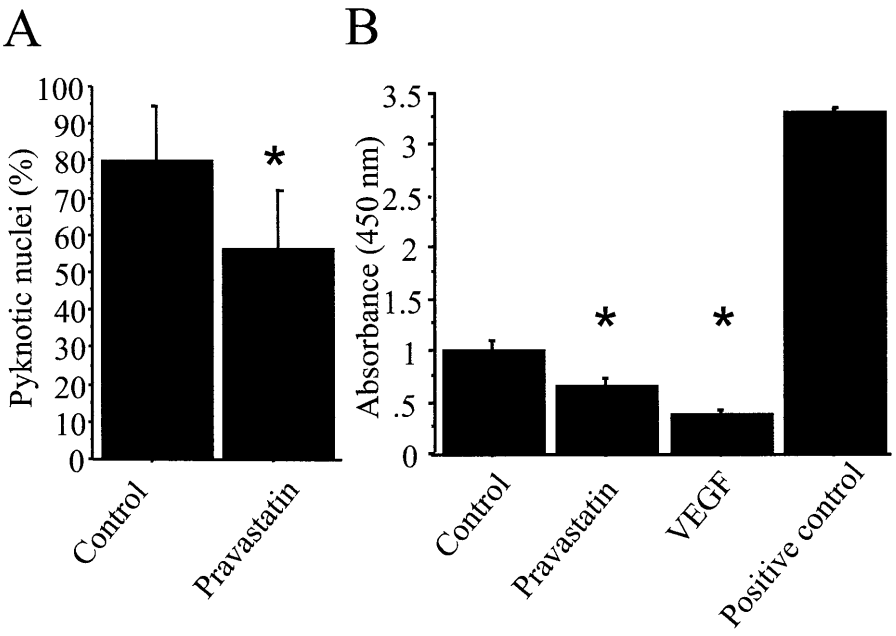

Fig. 3 Pravastatin inhibits EPC apoptosis induced by serum starvation
A; Percentage of pyknotic nuclei determined by DAPI staining, after serum starvation with or without pravastatin supplementation.
B; Light absorbance of apoptotic cells determined by cell death detection ELISA^{plus}. Positive control was DNA-histone complex to ensure experimental accuracy.

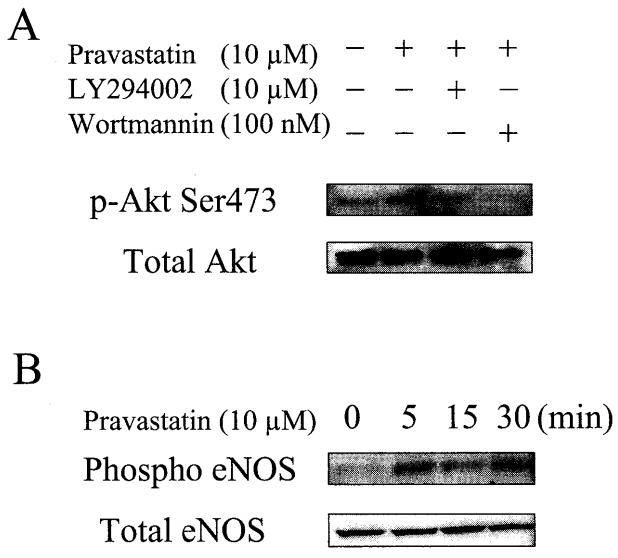

Fig. 4
A; Pravastatin rapidly activates Akt at 10 μM (5 min), and this effect was abrogated by wortmannin and LY294002, inhibitors of PI-3-kinase.
B; Time-course analysis of eNOS phosphorylation with 10 μM pravastatin treatment in EPCs. Pravastatin upregulates the phospho-eNOS signal in EPCs from 5 to 30 min after exposure.

more, the downstream signal of the PI-3-kinase/Akt kinase cascade of EPCs was investigated using an eNOS antibody. Pravastatin rapidly activated eNOS at the same concentration and this effect was detected for 30 min (Fig. 4B). The findings show that the effects of pravastatin on EPCs are mediated by eNOS activation through the PI-3-kinase/Akt pathway.

Effects of pravastatin on circulating EPC kinetics

As demonstrated in previous reports[12)14)31)], hydrophobic statins induce the mobilization of circulating EPCs. To assess whether pravastatin also induces EPC mobilization, mouse EPC culture assay using mouse PBMNCs was performed (Fig. 5A). Mouse PBMNCs collected at each time point (0, 5 and 14 days) after treatment with pravastatin (0.2 or 1 mg/kg/day) were applied to EPC culture assay. The number of cultured EPCs from peripheral blood increased significantly up to day 14, as compared with day 0 (day 5 and 14 vs day 0, fold increase; $*1.3 \pm 0.07$ and $*1.5 \pm 0.12$, $*p<0.05$, Fig. 5C). However, at the higher dose of pravastatin (1 mg/kg/day), the number of cultured EPCs was the same as that with vehicle (Fig. 5B).

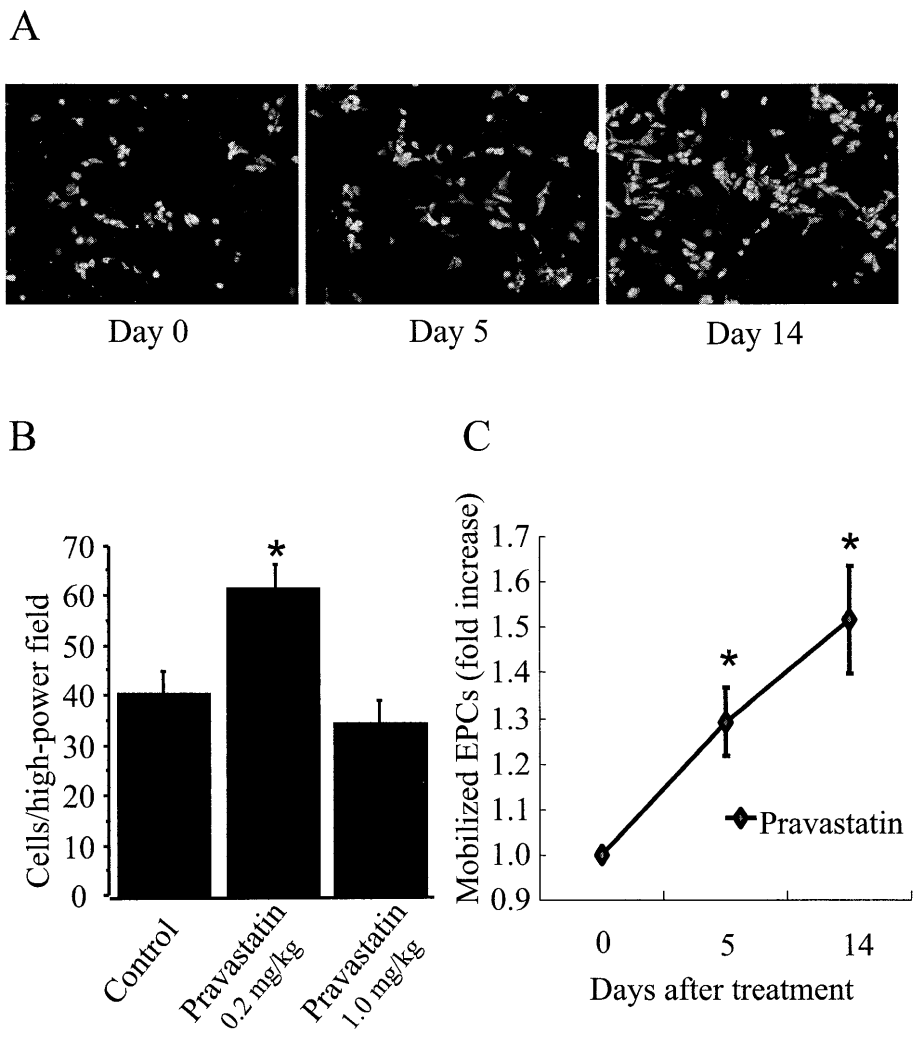

Fig. 5

A; Representative EPC images are shown as double-positive staining by AcLDL and BS-1 lectin.

B; Lower dose of pravastatin increases the number of cultured EPCs by 1.5-fold compared with controls at day 14. This effect was abolished at the higher dosage.

C; Pravastatin increases EPC number, depending on treatment duration.

Discussion

The impact of a hydrophilic statin (pravastatin) on EPC mobilization was similar to that of hydrophobic statins (data not shown). Thus, we hypothesized that pravastatin was taken up by human EPCs and exerted provasculogenic activity, similarly to hydrophobic statins.

In the present study, we demonstrated that pravastatin promotes EPC activity, i.e., migration, survival and mobilization, through the activation of the PI-3 kinase/Akt/eNOS pathway after intracellular uptake, although the uptake mechanism remains unknown.

It was previously considered that hydrophilic statin could not penetrate the lipid bilayers of the cell membrane in the absence of OATP2, except in hepatocytes that express a transporter[32]. However, another transporter, OATP-B, is broadly expressed in several human tissues, and reportedly transports hydrophilic statin into the intracellular space. Here, we demonstrated the cellular uptake of pravastatin by EPCs and a novel role for hydrophilic statins, namely the induction of vasculogenic potential in EPCs. Moreover, we documented that the hydrophilic statin activated eNOS via the PI-3 kinase/Akt pathway. Although the transporting peptide was

not detected in EPCs, pravastatin was taken up and demonstrated potent migration and antiapoptotic effects in EPCs at lower concentrations (1 μM). However, higher concentrations (10 μM) of pravastatin exerted the opposite effect (data not shown). This 'double-edged role' of statin is in line with recent publications[15)33)], i.e., atorvastatin induced a dose-dependent increase in EPC migration, with a maximal effect at 0.01 μM, but this promigratory effect was diminished at higher concentrations (1 μM). Thus, pravastatin exerts marked provasculogenic effects in EPCs, and these effects were similar to those of VEGF. Therefore, pravastatin may exert equivalent provasculogenic ability as hydrophobic statins, as a previous paper reported that a hydrophobic statin (simvastatin) had similar effects as VEGF[12)].

Furthermore, pravastatin upregulated the number of circulating EPCs in vivo, as assessed by EPC culture assay, similarly to hydrophobic statins. Interestingly, the fold increase over controls by treatment with pravastatin was similar to that reported in human subjects with atorvastatin[34)].

This study thus revealed the provasculogenic effects of pravastatin in vitro and in vivo. We could not directly confirm that circulating EPCs mobilized by pravastatin contributed into ischemic tissue due to the lack of a bone marrow transplantation model to investigate the incorporation of EPCs into ischemic tissue. However, it is well established that EPCs contribute to ischemic injury, and that the proportional contribution of angiogenesis and vasculogenesis to neovessel formation in ischemic tissue remains to be verified. It is thus possible that EPCs mobilized by pravastatin play a pivotal role in the recovery of neovessel formation in ischemic tissue, particularly considering previous reports[12)13)31)].

Because the mechanism of pravastatin uptake by EPCs remains unknown, there are several hypotheses to account for the physiological mechanisms. Firstly, pravastatin may be able to penetrate the lipid bilayer, but only when cell membrane permeability is high, such as during tissue or cellular ischemia. Secondly, as discussed in a previous paper, hydrophilic substances with a molecular weight (MW) of around 400 kDa may be able to penetrate the cell membrane through pore, i.e., passive diffusion[35)]. Considering this mechanism, pravastatin (450 kDa) may have been able to pass into the cells. Finally, on the surface of EPCs, there may another transporter of the OATP family, such as OATP-B, or a receptor that binds with pravastatin.

Hydrophobic statins, such as simvastatin, affect EPC proliferation[12)13)], whereas pravastatin did not [data not shown]. This implies the different molecular mechanisms for pravastatin and other hydrophobic statins.

Other hydrophobic statins are distributed in several tissues, and this may occasionally cause unexpected side effects in clinical settings. Pravastatin would be an ideal agent, if the compound has some type of selective tissue distribution due to a unique transport system.

In our experiments, pravastatin was shown to promote EPC migration, and cell survival in vitro via the PI-3 kinase/Akt/eNOS pathway in vitro, and to increase the number of circulating EPCs in vivo. eNOS activation is essential for EPC mobilization, as shown by Iwakura et al[28)], and the results of this study thus suggest that pravastatin has a potential role in the therapeutic neovascularization of ischemic tissue, even in patients with normal cholesterol levels.

Acknowledgements

This work was supported by Sankyo Co. Ltd. We thank H. Tsukamoto (Teaching and Research Support Center, Kanagawa, Japan) for technical assistance with the uptake experiments. We would also like to thank S. Ohta and M. Motoki for secretarial assistance.

References

1) **Pedersen TR, Kjekshus J, Berg K et al**: Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). 1994. Atheroscler Suppl **5**: 81–87, 2004
2) Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group. N Engl J Med **339**:

1349–1357, 1998
3) **Sacks FM, Pfeffer MA, Moye LA et al**: The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events Trial investigators. N Engl J Med **335**: 1001–1009, 1996
4) **Ridker PM, Rifai N, Pfeffer MA et al**: Inflammation, pravastatin, and the risk of coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events (CARE) Investigators. Circulation **98**: 839–844, 1998
5) Influence of pravastatin and plasma lipids on clinical events in the West of Scotland Coronary Prevention Study (WOSCOPS). Circulation **97**: 1440–1445, 1998
6) **Egashira K, Hirooka Y, Kai H et al**: Reduction in serum cholesterol with pravastatin improves endothelium-dependent coronary vasomotion in patients with hypercholesterolemia. Circulation **89**: 2519–2524, 1994
7) **Wassmann S, Laufs U, Baumer AT et al**: HMG-CoA reductase inhibitors improve endothelial dysfunction in normocholesterolemic hypertension via reduced production of reactive oxygen species. Hypertension **37**: 1450–1457, 2001
8) **Laufs U, La Fata V, Plutzky J et al**: Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. Circulation **97**: 1129–1135, 1998
9) **Corsini A, Bernini F, Quarato P et al**: Non-lipid-related effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. Cardiology **87**: 458–468, 1996
10) **Kureishi Y, Luo Z, Shiojima I et al**: The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. Nat Med **6**: 1004–1010, 2000
11) **Asahara T, Murohara T, Sullivan A et al**: Isolation of putative progenitor endothelial cells for angiogenesis. Science **275**: 964–967, 1997
12) **Llevadot J, Murasawa S, Kureishi Y et al**: HMG-CoA reductase inhibitor mobilizes bone marrow—derived endothelial progenitor cells. J Clin Invest **108**: 399–405, 2001
13) **Dimmeler S, Aicher A, Vasa M et al**: HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. J Clin Invest **108**: 391–397, 2001
14) **Walter DH, Rittig K, Bahlmann FH et al**: Statin therapy accelerates reendothelialization: a novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells. Circulation **105**: 3017–3024, 2002
15) **Urbich C, Dernbach E, Zeiher AM et al**: Double-edged role of statins in angiogenesis signaling. Circ Res **90**: 737–744, 2002
16) **Koga T, Shimada Y, Kuroda M et al**: Tissue-selective inhibition of cholesterol synthesis in vivo by pravastatin sodium, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor. Biochim Biophys Acta **1045**: 115–120, 1990
17) **Singhvi SM, Pan HY, Morrison RA et al**: Disposition of pravastatin sodium, a tissue-selective HMG-CoA reductase inhibitor, in healthy subjects. Br J Clin Pharmacol **29**: 239–243, 1990
18) **Abe T, Kakyo M, Tokui T et al**: Identification of a novel gene family encoding human liver-specific organic anion transporter LST-1. J Biol Chem **274**: 17159–17163, 1999
19) **Hsiang B, Zhu Y, Wang Z et al**: A novel human hepatic organic anion transporting polypeptide (OATP2). Identification of a liver-specific human organic anion transporting polypeptide and identification of rat and human hydroxymethylglutaryl-CoA reductase inhibitor transporters. J Biol Chem **274**: 37161–37168, 1999
20) **Nakai D, Nakagomi R, Furuta Y et al**: Human liver-specific organic anion transporter, LST-1, mediates uptake of pravastatin by human hepatocytes. J Pharmacol Exp Ther **297**: 861–867, 2001
21) **Tamai I, Nezu J, Uchino H et al**: Molecular identification and characterization of novel members of the human organic anion transporter (OATP) family. Biochem Biophys Res Commun **273**: 251–260, 2000
22) **Kullak-Ublick GA, Ismair MG, Stieger B et al**: Organic anion-transporting polypeptide B (OATP-B) and its functional comparison with three other OATPs of human liver. Gastroenterology **120**: 525–533, 2001
23) **Kobayashi D, Nozawa T, Imai K et al**: Involvement of human organic anion transporting polypeptide OATP-B (SLC21A9) in pH-dependent transport across intestinal apical membrane. J Pharmacol Exp Ther **306**: 703–708, 2003
24) **Takahashi T, Kalka C, Masuda H et al**: Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med **5**: 434–438, 1999
25) **Asahara T, Takahashi T, Masuda H et al**: VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. Embo J **18**: 3964–3972, 1999
26) **Iwaguro H, Yamaguchi J, Kalka C et al**: Endothelial progenitor cell vascular endothelial growth factor gene transfer for vascular regeneration. Circulation **105**: 732–738, 2002
27) **Yamaguchi J, Kusano KF, Masuo O et al**: Stromal cell-derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization. Circulation **107**: 1322–1328, 2003
28) **Iwakura A, Luedemann C, Shastry S et al**: Estrogen-mediated, endothelial nitric oxide synthase-dependent mobilization of bone marrow-derived endothelial progenitor cells contributes to reendothelialization after arterial injury. Circulation **108**: 3115–3121, 2003
29) **Seeger FH, Haendeler J, Walter DH et al**: p38 mitogen-activated protein kinase downregulates endothelial progenitor cells. Circulation **111**: 1184–1191, 2005
30) **Kalka C, Masuda H, Takahashi T et al**: Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc

Natl Acad Sci U S A **97**: 3422–3427, 2000
31) **Landmesser U, Engberding N, Bahlmann FH et al**: Statin-induced improvement of endothelial progenitor cell mobilization, myocardial neovascularization, left ventricular function, and survival after experimental myocardial infarction requires endothelial nitric oxide synthase. Circulation **110**: 1933–1939, 2004
32) **Kaneta S, Satoh K, Kano S et al**: All hydrophobic HMG-CoA reductase inhibitors induce apoptotic death in rat pulmonary vein endothelial cells. Atherosclerosis **170**: 237–243, 2003
33) **Vincent L, Chen W, Hong L et al**: Inhibition of endothelial cell migration by cerivastatin, an HMG-CoA reductase inhibitor: contribution to its anti-angiogenic effect. FEBS Lett **495**: 159–166, 2001
34) **Vasa M, Fichtlscherer S, Adler K et al**: Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. Circulation **103**: 2885–2890, 2001
35) **Pappenheimer JR, Renkin EM, Borrero LM**: Filtration, diffusion and molecular sieving through peripheral capillary membranes; a contribution to the pore theory of capillary permeability. Am J Physiol **167**: 13–46, 1951

血管内皮前駆細胞の血管新生作用に対する水溶性スタチン（プラバスタチン）の影響

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脂溶性 HMG-CoA リダクターゼ・インヒビター（脂溶性スタチン）による血管内皮前駆細胞（EPC）動員，治療的血管形成作用改善効果は多数の報告がされている．しかしながら水溶性スタチン（プラバスタチン）の EPC に対する作用は充分に解明されていない．本研究により，水溶性スタチンが EPC 内に取り込まれ，細胞内シグナルを刺激することで EPC 動態に作用することを明らかとする．

^{14}C 標識プラバスタチンにより，HepG2 細胞を対照群として EPC 内へのプラバスタチン取り込みを測定した結果，EPC 群は有意にプラバスタチンを細胞内に取り込んだ（$*29.15 \pm 2.80$ vs 7.82 ± 0.47 count/min/mg protein, $^{*}p<0.0001$）．EPC の遊走能測定実験において，プラバスタチン群は対照群と比較して，有意に遊走能を増加させた．また，抗アポトーシス作用測定実験において，プラバスタチン群は対照群と比較して有意にアポトーシスを抑制していた．更に，ウェスタンブロットを行ったところ，EPC 内においてプラバスタチンは PI-3-kinase/Akt 経路を刺激することにより，eNOS のリン酸化を行うことが判明した．この反応は PI-3/Akt 阻害剤により消失することにより，PI-3-kinase/Akt 経路依存性であることが確かめられた．In vivo において，プラバスタチンをマウスに投与したところ，プラバスタチンは対照群と比較して投与時間依存性に末梢血液中の EPC 数を増加させることが判明した．

これらの結果により，水溶性スタチンであるプラバスタチンは，EPC 内の PI-3-kinase/Akt/eNOS 経路により，EPC 遊走能・抗アポトーシス作用を活性化することが明らかとなった．