# 3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor (pravastatin) inhibits endothelial cell proliferation dependent on $G_1$ cell cycle arrest

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**3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors have been developed as lipid-lowering drugs, and are well recognized to reduce morbidity and mortality from coronary artery disease. Several recent experimental studies have focused on the inhibitory effects of HMG-CoA reductase inhibitor on tumor cell growth *in vitro* and *in vivo*, dependent on a direct effect on cancer cells. In the present study, we aimed to investigate the potential anti-angiogenic effect of pravastatin and its mechanism of action. Using human umbilical vein endothelial cells (HUVECs) as a model of angiogenesis, we investigated the effect of pravastatin on the various steps of angiogenesis, including endothelial cell proliferation and adhesion to extracellular matrix proteins. Pravastatin induced a dose-dependent decrease in the proliferative activity of endothelial cells, which was dependent on the cell cycle arrest to the $G_1$ phase and not on cell apoptosis. $G_1$ arrest was due to the decrease of cyclin D, cyclin E and cyclin-dependent kinase 2 levels. In addition, pravastatin inhibited tube formation on Matrigel and adhesion to extracellular matrix, but did not affect matrix metalloproteinase production. The present results demonstrate the anti-angiogenic activity of pravastatin and its potential use as an anticancer drug is suggested.**
***Anti-Cancer Drugs*** **15:625–632 © 2004 Lippincott Williams & Wilkins.**

**Anti-Cancer Drugs** 2004, **15**:625–632

**Keywords: angiogenesis inhibitors, cell cycle, cell proliferation, endothelial cells, statins**

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Sponsorship: This study was supported partly by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, partly by a grant from the Ministry of Health, Labor and Welfare of Japan, and partly by Sankyo, Japan.

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**Received** 10 February 2004 **Revised form accepted** 24 March 2004

## Introduction

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase is considered the major regulatory enzyme of the mevalonate metabolic pathway. HMG-CoA reductase inhibitors, i.e. statins, are reversible inhibitors of the rate-limiting step in cholesterol biosynthesis and are generally used for the treatment of hypercholesterolemia [1,2].

In addition to regulation of cholesterol metabolism, many recent experimental studies have focused on the effects of HMG-CoA on tumor cell growth *in vitro* and *in vivo*, and have demonstrated the perturbation of cell cycle pathways dependent on the induction of cyclin-dependent kinase inhibitors (CKIs), i.e. p21 and p27 [3–8].

However, the effect of statins on angiogenesis is poorly understood. Neo-vascularization, or angiogenesis, is a common attribute of tumors, and is crucial for the formation, growth and dissemination of cancer. Tumor cells constitute a population of genetically unstable cells, with an ability to mutate and develop resistance to chemotherapeutic drugs, whereas endothelial cells (ECs) that supply tumors with a vasculature are a stable population of cells, which do not change despite mutational events in tumor cells. Because angiogenesis is infrequent in the adult, there is the potential to develop very specific anti-cancer therapies with minimal toxicities except during times of wound healing, inflammation, ovulation, pregnancy or ischemia [9].

In the present study, we aimed to investigate the potential anti-angiogenic effect of pravastatin. For this purpose, we used an *in vitro* model of angiogenesis consisting of human umbilical vein endothelial cells (HUVECs), and clearly demonstrated that pravastatin inhibited EC proliferation by induction of cell cycle arrest to the $G_1$ phase and should be considered a promising new anti-angiogenic agent.

0959-4973 © 2004 Lippincott Williams & Wilkins DOI: 10.1097/01.cad.0000131680.83518.91

## Material and methods

### HMG-CoA reductase inhibitor

The HMG-CoA reductase inhibitor, pravastatin, was a kind gift from Sankyo (Tokyo, Japan).

### HUVEC isolation and culture

Umbilical cords were obtained from normal pregnant individuals at delivery, after informed consent was obtained. HUVECs were isolated from the umbilical cord blood vessels, as previously described [10]. Briefly, the umbilical vein was cannulated at the both edges and 0.2% collagenase I in $Ca^{2+}$/$Mg^{2+}$-free phosphate-buffered saline [PBS(–)] was added to the lumen of the vessel. The umbilical cord was incubated at 37°C for 20 min, and then the PBS(–) containing collagen was recovered and centrifuged to obtain the isolated cells. Cells were washed twice with PBS(–), suspended in complete medium consisting of MCDB-151 medium (Sigma-Aldrich Japan, Tokyo, Japan) supplemented with 15% fetal calf serum (FCS), 2.0 ng/ml of acidic fibroblast growth factor (aFGF) and 5 μg/ml of heparin, and seeded in culture dishes previously coated with 0.1% gelatin.

Cells were routinely cultured in complete medium in an atmosphere of 5% $CO_2$ at 37°C, passaged by trypsinization and used up to 10 passages for the experiments.

### *In vitro* proliferation assay

HUVECs were seeded at $3 \times 10^3$/well in 96-well flat-bottomed plates in MCDB-151 medium containing 15% FCS and allowed to adhere for 2 h. The bottom of each well was previously coated with 0.1% gelatin. Then, medium containing differing concentrations of pravastatin diluted in PBS was added to give the final concentrations of 0, 50, 100, 200 and 500 μM. The medium was renewed every other day from the beginning of treatment. After 3 and 5 days culture in the presence of pravastatin, the proliferative activity was determined by MTS assay (CellTiter 96 Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI), which monitors the number of viable cells, according to manufacturer's instructions. Briefly, MTS solution was added at 20 μl/well and, after 4 h culture, the conversion of MTS to formazan was measured in a plate reader at 490 nm. All experiments were performed in triplicate wells and the proliferative activity was calculated as the ratio of each experimental condition to the control one. In addition, to confirm that the effect of pravastatin on ECs was mediated through the HMG-CoA reductase, ECs were pre-treated with farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), which are the downstream products of mevalonic acid, prior to pravastatin.

### Detection of apoptosis

Under subconfluent conditions of HUVECs, MCDB medium containing differing concentrations of pravastatin was added to give the final concentrations of 0, 100, 200 and 500 μM. After 48 h culture, cells were harvested by trypsinization, washed twice in PBS(–), and then staining of cells with the combination of Annexin-V/FITC and propidium iodine (PI) (Annexin-V: FITC Apoptosis Detection Kit; BD PharMingen, San Jose, CA) was performed according to the manufacturer's instructions. Briefly, cells were suspended in the supplied binding buffer at a concentration of $1 \times 10^6$/ml and 100 μl ($1 \times 10^5$ cells) was transferred to the test tube. HUVECs of each group were incubated with saturating concentrations of Annexin-V/FITC (Alexis, Montreal, Canada) and PI (Sigma-Aldrich Japan) for 10 min at 4°C in the dark. After addition of 400 μl of the binding buffer to each sample, samples were immediately analyzed using a flow cytometer (FACSCalibur; Becton Dickinson, San Jose, CA). This method allows the distinction between the early (Annexin-V/$FITC^+$/$PI^-$) and late (Annexin-V/$FITC^+$/$PI^+$) apoptosis cells, but in the present study both subpopulations were considered to represent the total fraction of apoptotic cells.

### Detection of cell cycle distribution by flow cytometry

Subconfluent HUVECs were treated with differing concentrations of pravastatin diluted in PBS, to give the final concentrations of 0, 10, 200 and 500 μM, for 48 h in an atmosphere of 5% $CO_2$ at 37°C. Then 5-bromo-2′-deoxyuridine (BrdU), at the final concentration of 5 μg/ml, was added to the medium and the cells incubated for a further 1 h under the same conditions. HUVECs were then harvested by trypsinization, washed twice with PBS(–) and fixed with ethanol. After staining with PI and FITC-conjugated anti-BrdU antibody (BD PharMingen), the percentage of cells in each cell cycle phase was measured by the FACSCalibur.

### Western blotting

HUVECs were seeded on 0.1% gelatin-coated 150-mm plastic dishes and grown to subconfluence in complete medium. Then, medium was removed by aspiration, and medium containing different concentrations of pravastatin (0, 100, 200 and 500 μM) was added and ECs cultured for an additional 48 h. The medium was then removed, and the adherent cell monolayers washed twice with PBS(–) and lysed with 0.5 ml of Tris–saline (50 mM Tris–HCl, pH 7.6 and 150 mM sodium chloride) containing various protease inhibitors (1 mM EGTA, 0.1 mM diisopropyl fluorophosphates, 0.5 mM phenylmethylsulfonyl fluoride, 1 mg/ml Na-*p*-tosyl-L-lysine chloromethyl ketone, 1 mg/ml antipain, 0.1 mg/ml pepstain and 1 mg/ml leupeptin) and 1% Triton-X for 1 h (on ice). ECs were then harvested using a cell scraper, centrifuged at 15 000 r.p.m. for 15 min, and the clear supernatant collected and used as the cell protein extract.

SDS–PAGE was performed as described previously [11] using a Laemmli buffer system and 12.5% polyacrylamide

resolving slab gels. Proteins were then transferred electrophoretically to polyvinylidine difluoride (PVDF) membranes (Immunobilon P; Millipore, Bedford, MA). The PVDF membrane was first equilibrated in transfer buffer (192 mM glycine, 25 mM Tris and 20% methanol, pH 8.3). The samples were reduced with *2%* 2-mercaptoethanol (2-ME). Blotting was carried out at 150 V for 1 h (4°C). After blotting, the remaining reactive sites of the PVDF membrane were blocked by incubating the sheets in TBS containing 10% skim milk. The same buffer containing 10% calf serum was used to dilute either antisera or purified antibodies. The membrane strips were incubated with the primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight (16–20 h) at ambient temperature on a shaker. Primary antibodies used were pRb, p21, p27, cyclin E, cyclin-dependent kinase (CDK) 2 and CDK6, mouse monoclonal antibodies, at a dilution 1:100. Rabbit polyclonal antibodies, Cyclin D and CDK4 were used at a 1:1000 dilution of biotinylated anti-mouse or anti-rabbit IgG (Vector, Burlingame, CA). After 1 h, the strips were washed an additional 3 times and then incubated with a freshly prepared avidin–biotin complex (ABC Kit; Vector) solution for 30 min. After washing 3 times, color development was carried out with a solution of diaminobenzidine (DAB Kit; Dako, Carpinteria, CA).

#### Adhesion of ECs to the extracellular matrix (ECM)

The ECMs used were collagens type I, III and IV, fibronectin, and laminin. Collagens were used at 30 μg/ml, and fibronectin and laminin at 10 μg/ml. Gelatin (0.2% in PBS) was used as the control.

The 96-well flat-bottomed plates were coated with each of the ECMs for overnight at 4°C. After blocking for non-specific binding with a buffer consisting of RPMI 1640 medium (Sigma-Aldrich Japan) supplemented with 0.2% bovine serum albumin (BSA; Sigma-Aldrich Japan), the adhesion assay was performed as previously described [12]. Briefly, after the treatment with or without pravastatin, ECs were labeled by incubation with calcein (10 μg/ml, Calcein-AM solution; Dojindo, Kumamoto, Japan) for 1 h and the cells resuspended in assay buffer at $6 \times 10^5$/ml. Calcein-labeled ECs were then seeded at 100 μl/well on the ECM-coated plate. The plate was incubated at 37°C in an atmosphere of 5% $CO_2$ for 5 min to allow ECs to sediment and adhere to the ECM. Then the wells were washed twice with assay buffer to remove the non-adherent cells and the fluorescence intensity of the adherent cells measured in a fluorometer (TerascanVP; Minervateck, Tokyo, Japan).

#### Gelatin zymography assay

To investigate the effect of pravastatin on the production of matrix metalloproteinases (MMPs), gelatin zymography was performed as described [11]. Briefly, HUVECs were treated with pravastatin (0, 100 and 250 μM) in MCDB medium containing 15% FCS (complete medium) for 48 h. Then, cells were harvested by trypsinization, washed 3 times in PBS, and $1 \times 10^7$ cells were resuspended in serum-free DMEM:F12 (1:1 ratio) medium (both from Sigma-Aldrich Japan) and incubated for another 4 h. The cell supernatants were then collected, centrifuged to eliminate the contaminating cells and debris, and concentrated 25 times in a Centricon concentrator 10 (Amicon, Beverly, MA) by centrifugation. The samples were then subjected to SDS–PAGE in 10% acrylamide gels containing 0.1% gelatin. After washing in a 2% Triton X-100 solution (washing buffer) for 1 h at room temperature, the gels were transferred to the incubation buffer containing $Zn^{2+}$ and incubated overnight at room temperature to allow the MMPs to degrade gelatin. The gels were stained for 30 min with Coomassie blue for visualization of the MMPs.

#### Capillary-like tube formation

The formation of vascular-like structures by HUVECs on the basement membrane matrix, using Matrigel basement membrane matrix (Becton Dickinson, Bedford, MA), was assessed. A six-well flat-bottomed plate was coated with Matrigel (100 μl/well) and HUVECs seeded at $1 \times 10^5$cells/well in complete medium containing differing concentrations of pravastatin (0, 100, 200 and 500 μM). After incubation at 37°C for 24 h, the wells were observed using a phase contrast microscope. Images were captured with a digital camera.

#### Statistical analysis of the data

For the statistical analysis of the data, the Student's *t*-test was used and $p < 0.05$ was considered statistically significant.

## Results

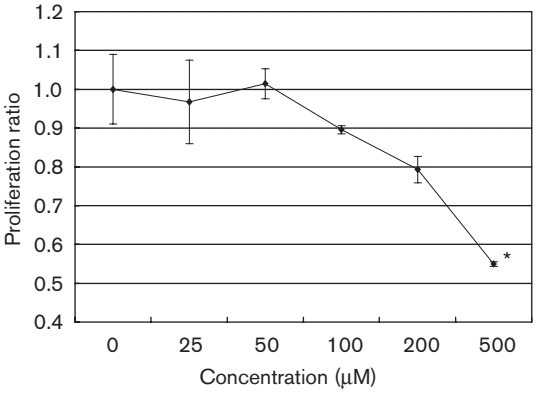
#### Pravastatin inhibited the proliferative activity of ECs

As shown in Figure 1, pravastatin inhibited the proliferative activity of HUVECs, in a dose-dependent manner. Pravastatin at 50 μM had no inhibitory effect, but at 100 μM, an approximately 10% inhibitory effect was observed. With the highest concentration tested (500 μM), an up to 45% inhibition was observed after 5 days treatment. The inhibitory effect of pravastatin could be significantly reversed by GGPP (20 μM), but only partially by FPP (20 μM) (Fig. 2).

#### Pravastatin does not induce ECs apoptosis

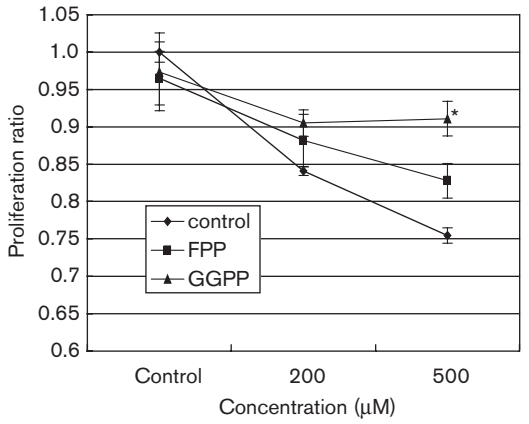
To determine whether the inhibitory effect of pravastatin on the proliferative activity of ECs was dependent on cell death, we performed the apoptosis detection assay of pravastatin-treated ECs using flow cytometry and compared with the untreated cells. The upper-right and the lower-right regions of the quadrant statistical chart represent cells in apoptosis. As shown in Figure 3, there

**Fig. 1**




The proliferative activity of ECs, treated without or with various concentrations of pravastatin, was assessed by MTS assay, which measures the dehydrogenase activity in cell culture medium. Pravastatin caused a dose-dependent inhibitory effect on the proliferation of ECs. *$p<0.05$.

**Fig. 2**



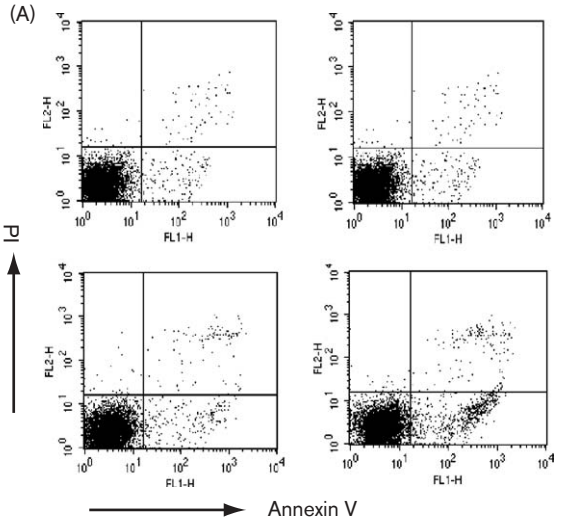

GGPP (20 μM), one precursor of cholesterol, strongly inhibited the inhibitory effect of pravastatin on EC proliferation, whereas FPP (20 μM), another precursor, had only a partial inhibitory effect. *$p<0.05$.

is no significant difference in rate of apoptotic cells between ECs treated with pravastatin at either 100 or 200 μM, compared to control untreated cells. The population of early apoptotic cells increased only with the highest dose of pravastatin tested (500 μM). Thus, the anti-proliferative effect of pravastatin on ECs is not mostly dependent on apoptosis induction.

### Pravastatin induced cell cycle arrest of ECs

To determine whether the anti-proliferative effect of pravastatin on ECs was mediated through an alteration of the cell cycle, we analyzed the cell cycle distribution of

**Fig. 3**




(B)

| Pravastatin (μM) | 0 | 100 | 200 | 500 | |
|---|---|---|---|---|---|
| Apoptosis | 2.5 | 2.6 | 3.6 | 9.7 | |
| Necrosis | 0.2 | 0.5 | 0.3 | 0.3 | (%) |

Cell apoptosis was analyzed by flow cytometry, after Annexin V and PI double-staining of ECs treated without or with pravastatin at different doses. Pravastatin caused an increase in the percentage of apoptotic ECs only at the highest dose tested (500 μM).

pravastatin-treated ECs and compared with untreated cells. As determined by flow cytometry, treatment with pravastatin caused a dose-dependent inhibition of the progression from the $G_1$ to S phase in ECs, which resulted in a clear increase of the percentage of cells in the $G_1$ phase compared with control cells (Fig. 4). These results are consistent with the results of the proliferation assay.

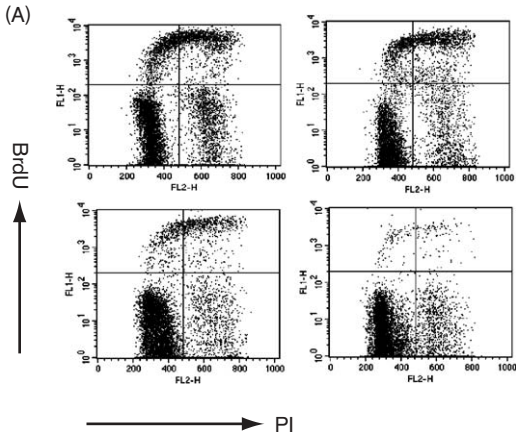
### Expression of cell cycle regulators by Western blotting

Next, we examined the pattern of expression of the key cell cycle regulators, i.e. pRb, p21, p27, CDK2, CDK4, CDK6, cyclin D and cyclin E, in pravastatin-treated ECs and compared with untreated cells. Each cell cycle regulatory protein was constitutively expressed in ECs. Pravastatin treatment decreased the total pRb expression and, in particular, its phosphorylated form (Fig. 5). Also, the levels of CDK2, cyclin D and cyclin E decreased in a dose-dependent manner. However no significant effect on p21, p27, CDK4 and CDK6 levels was observed.

### Pravastatin did not affect the production of MMPs by ECs

As shown in Fig. 6, high levels of MMP-2 and low levels of MMP-9 were detected in the supernatants of ECs by gelatin zymography, but pravastatin treatment had no

**Fig. 4**

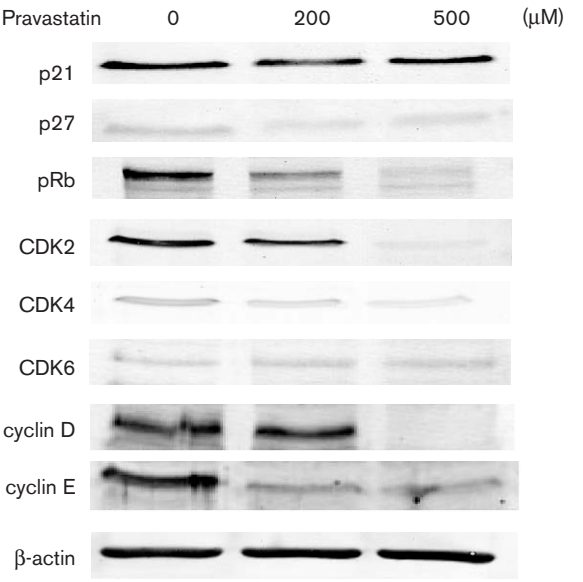



(B)

| Phase \ PRV (μM) | 0 | 100 | 200 | 500 | |
|---|---|---|---|---|---|
| S | 24.3 | 18.6 | 10.7 | 2.01 | |
| $G_1$ | 59.6 | 66.5 | 77.3 | 86.1 | |
| $G_2$,M | 16.2 | 14.9 | 11.9 | 11.9 | (%) |

Cell cycle distribution of ECs treated without or with different doses of pravastatin, analyzed in a flow cytometer. Treatment with pravastatin for 48 h resulted in a dose-dependent accumulation of cells in the $G_1$ phase, with a corresponding decrease in the cells in S phase. PRV: pravastatin.

**Fig. 5**




ECs were cultured for 48 h without or with pravastatin (200 and 500 μM), then harvested and the protein lysates subjected to 10% SDS–PAGE for analysis of the main cell cycle regulators. The expression levels of CKIs, i.e. p21 and p27, were not affected, but the expression levels of CDK 2, cyclin D and E decreased in a dose-dependent manner by pravastatin treatment. Phosphorylated pRb was also reduced dose dependently.

significant effect on the active and proenzyme forms of MMPs.

### Pravastatin decreased the adhesion of ECs to ECM

The effect of statin on the ability of ECs to bind ECM was evaluated under static conditions *in vitro*. Pravastatin caused a dose-dependent inhibition on the ability of ECs to bind collagens I, III and laminin, and the effect was significant with pravastatin at the dose of 200 μM or higher (Fig. 7).

### Pravastatin inhibited capillary-like tube formation

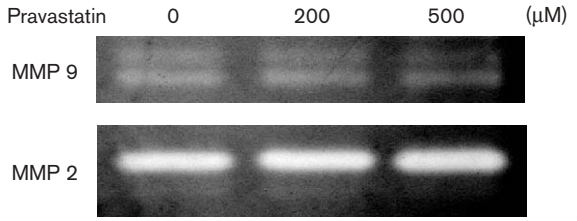
To examine the effect of statins on the ability of ECs to form vascular structures *in vitro*, a Matrigel tube formation assay was performed in the absence or presence of pravastatin. Capillary-like tubular formation was clearly observed in control and pravastatin-treated (at 200 μM) ECs, but not in ECs treated with the highest dose of pravastatin (500 μM) (Fig. 8).

## Discussion

HMG-CoA reductase is the rate-limiting enzyme in the cholesterol biosynthetic pathway and statins, which are potent HMG-CoA reductase inhibitors, are used in the treatment of hypercholesterolemia [1,13]. Recently, HMG-CoA reductase inhibitor has been reported to reduce the risk of cancer and is suggested to act as a cancer-preventive agent. Several studies described the suppression of cancer cells proliferation by statins. Lovastatin, a kind of statin, has been shown to inhibit proliferation of pancreatic cancer cell lines *in vitro* and *in vivo* [14], and of a breast cancer cell line *in vitro* [15]. Kawata *et al.* designed a randomized clinical trial, with death as the primary endpoint, to investigate whether pravastatin affected the survival of patients with advanced unresectable hepatocellular carcinoma and demonstrated that patients in the pravastatin group had a significantly longer survival rate than control patients [16]. These facts suggest a protective effect of statins on cancer progression, but the precise mechanisms of the anti-cancer effects of statins still remains to be elucidated.

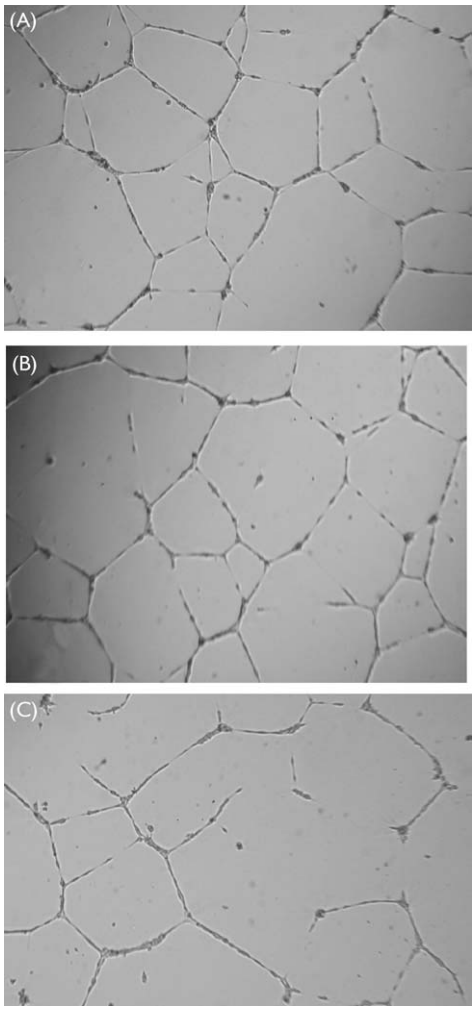
Angiogenesis, the formation of new microvessels, is fundamental for tumor growth of solid tumors, by supplying nutrients to cancer cells [17–19]. During angiogenesis, endothelial cells emerge from the quiescent state and undergo rapid proliferation. Consequently, the selective targeting of tumor vascular endothelium is a promising strategy to combat cancer, since it will interrupt the oxygen and nutrient supply to tumor cells.

**Fig. 6**



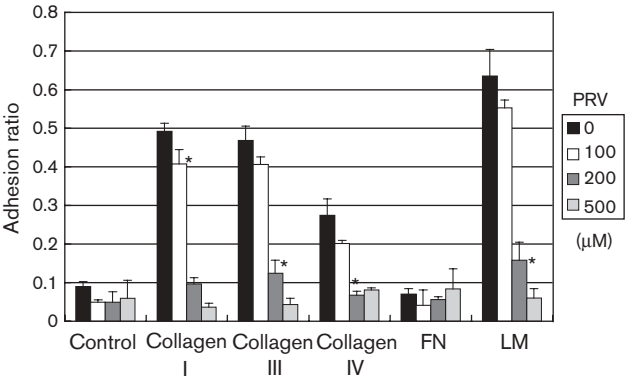

HUVECs were incubated for 48 h with or without pravastatin (200 and 500 μM) in protein-free media. Conditioned media were collected and the MMP activity was measured by gelatin zymography. ECs produced high levels of MMP-2 and moderate levels of MMP-9. Pravastatin treatment, however, did not affect the production of either MMPs.

**Fig. 7**




Representative microphotographs of vascular-like tube formation on Matrigel. The formation of vascular-like structures was assessed on ECs without (A) or with treatment with pravastatin at 200 (B) or 500 (C) μM. Tube-like structure formation was clearly seen in untreated ECs as well as ECs treated with pravastatin at 200 μM, but at the highest dose of pravastatin only incomplete formations were observed. Tube formation was determined by phase microscopy at × 200 original magnification.

**Fig. 8**




The ability of ECs to adhere to various ECM proteins was assessed by an adhesion assay using fluoro-labeled ECs. Pravastatin dose-dependently inhibited EC adhesion to ECM, and significant inhibition was observed for collagens I and III, and laminin. $*p < 0.05$.

The effects of HMG-CoA reductase inhibitor on endothelial cells have been reported in terms of atherosclerosis, but presently little is known on the effect of statins on angiogenesis. On atherosclerotic endothelium, cholesterol-lowering therapy with pravastatin seems to improve the endothelium-dependent coronary vasomotion, which may possibly contribute to the improvement of myocardial perfusion as well as the regression of coronary atherosclerosis [20]. Part of this anti-atherogenic effect of pravastatin seems to be due to an improvement in the resistance of atherosclerogenic lipoproteins to oxidation [21].

In this study, we aimed to examine the effect of pravastatin on angiogenesis, using HUVECs as a model. Pravastatin induced a dose-dependent inhibition of EC proliferation. This inhibition was not dependent on EC apoptosis, but on the cell cycle arrest. Pravastatin induced a cell cycle arrest at the $G_1$ phase, dependent on a decrease in the expression of CDK 2, cyclin D and cyclin E. Phosphorylation of pRb, which is necessary for the progression through $G_1$, is regulated primarily by the cyclin D/CDK4/CDK6 complex, while the cyclin E/CDK 2 complex regulates the passage of cells from late $G_1$ to S phase and further contributes to pRb hyper-phosphorylation [22]. The pravastatin-induced cell cycle arrest, therefore, seems to be dependent on the down-regulation of phosphorylated pRb in the late $G_1$ phase. The anti-proliferative effect of pravastatin was almost completely reversible by GGPP, but only partially by FPP. This indicates that this inhibitory effect of pravastatin is mainly through the inhibition of Rho A geranylation. Different from our findings, Vincent *et al.* showed the induction of $G_1$ arrest on human microvascular

endothelial cells (HMEC-1), which was dependent on increasing p21 and Rho A inactivation [23] by cerivastatin. Some other reports, however, have described the induction of apoptosis of HUVECs and human dermal microvascular endothelial cells by statins. This apoptotic effect was dependent on the interference with protein geranylgeranylation and membrane localization of Rho A [24–27]. On the contrary, another study reported on a protective effect of statin, i.e. simvastatin, on the apoptotic death of ECs [25].

The process of neovascularization is dependent not only on EC proliferation, but also adhesion to the ECM, migration and tubular formation are important steps [28]. The cell–matrix interaction is the initial and crucial step in the angiogenic cascade. Endothelial cells need to adhere to the ECM proteins for cell spreading and, through an 'in–out signaling' mediated by integrins, they proliferate. Pravastatin, by causing a decrease in the ability of ECs to bind ECM, cause the disruption of this cell–matrix interaction, with consequent inhibition of EC proliferation. In the process of cell migration, ECs depend on synthesis of MMPs, enzymes essential for the degradation of ECM proteins. HMG-CoA reductase inhibitor, fluvastatin, inhibited MMP-9 activity in human monocyte-derived macrophages [29]. In the present study, however, pravastatin did not affect the production of MMPs by ECs, which is one of the important steps in the process of neovascularization. We also investigated the effect of pravastatin on capillary-like tube formation on Matrigel and found a significant inhibitory effect. Similar to ours, Park *et al.* showed that simvastatin inhibited the development of capillary-like structures by human microvascular endothelial cells cultured on three-dimensional collagen gels [26]. Contradicting this and our findings, Kureishi *et al.* and Chen *et al.* reported that, like vascular endothelial growth factor, statins promoted the formation of capillary-like tubes [25,30]. Simvastatin also promoted capillary formation in the ischemic limb. The discrepancies between these findings and ours could be due to the differences of EC origin, kind of statins and the concentrations of statins.

In humans, the maximum achievable serum level of pravastatin, after oral administration (20 mg/day), is 16.9 ng/ml (0.038 μM) (data provided by Sankyo). In our *in vitro* experiments, however, the dose of pravastatin necessary to obtain anti-proliferative effects on ECs was more than 100 μM. Therefore, to develop pravastatin as an anti-angiogenic agent in clinical practice, the application of a drug delivery system to improve drug absorption, or eventually the use of either i.v. or intratumoral injections is necessary and should be considered. In addition, there is need to investigate the possible side-effects dependent on the administration of such high doses.

In conclusion, for the first time we provided evidence that pravastatin at high doses is a promising anti-angiogenic drug, by causing suppression of endothelial cell proliferation through induction of cell cycle arrest, as well as inhibiting capillary-like tube formation. These anti-angiogenic properties, in addition to their inhibitory effect on cancer cell proliferation, make statins an attractive anti-tumor agent and their clinical applicability to cancer patients should be carefully considered.

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