Original Article

Stimulatory Effects of Low-Dose 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Inhibitor Fluvastatin on Hepatocyte Growth Factor–Induced Angiogenesis: Involvement of p38 Mitogen-Activated Protein Kinase

Akira URUNO[1)], Akira SUGAWARA[2)], Masataka KUDO[1)], Fumitoshi SATOH[1)], Akiko SAITO[3)], and Sadayoshi ITO[1)]

Therapeutic angiogenesis has received much attention for its potential benefits in ischemic vascular disorders. Recently, the clinical application of hepatocyte growth factor (HGF) for therapeutic angiogenesis has become well known. Statins have also been reported to promote angiogenesis and ameliorate ischemic conditions. In the present study, we examined the effects of fluvastatin on HGF-induced angiogenesis using a human umbilical vein endothelial cell (HUVEC)/normal human dermal fibroblast (NHDF) co-culture system. The HGF-induced angiogenesis was augmented by fluvastatin at low dose, but it was decreased at high dose. Although fluvastatin increased vascular endothelial growth factor expression in NHDFs, it was observed only at a high dose. Low-dose fluvastatin decreased the HGF-induced p38 mitogen-activated protein kinase (MAPK) phosphorylation (Thr-180/Tyr-182) and HUVEC apoptosis in the presence of HGF. SB203580, a p38 MAPK inhibitor, ameliorated anisomycin (a p38 MAPK activator)-induced angiogenesis suppression in the presence of HGF. Moreover, the augmentation of the HGF-induced angiogenesis by fluvastatin was abrogated by the p38 MAPK inhibitors, SB203580, SB202190, and FR167653. High-dose fluvastatin decreased Akt phosphorylation (Ser-473) and HUVEC proliferation, and it increased $p27^{kip1}$ in HUVECs. Interestingly, fluvastatin decreased the mRNA expression of integrins and tissue inhibitor of metalloproteinases (TIMPs) in HUVECs. Our data therefore indicate that the stimulatory effects of low-dose fluvastatin on the HGF-induced angiogenesis are mediated by its inhibitory effects on p38 MAPK phosphorylation induced by HGF, which may result in the suppression of EC apoptosis. High-dose fluvastatin inhibits Akt phosphorylation and HUVEC proliferation, and it increases $p27^{kip1}$, which may result in its inhibitory effects on angiogenesis. In addition, integrins and TIMPs are candidates for angiogenesis regulation by fluvastatin. (*Hypertens Res* 2008; 31: 2085–2096)

***Key Words*: statins, angiogenesis, p38 mitogen-activated protein kinase, hepatocyte growth factor**

From the [1)]Division of Nephrology, Endocrinology, and Vascular Medicine, Department of Medicine, [2)]Department of Advanced Biological Sciences for Regeneration, and [3)]Department of Pediatrics, Tohoku University Graduate School of Medicine, Sendai, Japan.

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (No. 17790545 for A.U., No. 16590898 for A.S., and No. 17390245 for S.I.), Grants-in-Aid from the Ministry of Health, Labour and Welfare of Japan (for A.S.), Takeda Science Foundation (for A.S.), and the 21st Century Centers of Excellence (COE) Program Special Research Grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Address for Reprints: Akira Sugawara, M.D., Ph.D., Department of Advanced Biological Sciences for Regeneration, Tohoku University Graduate School of Medicine, 2–1, Seiryo-machi, Aoba-ku, Sendai 980–8575, Japan. E-mail: akiras2i@mail.tains.tohoku.ac.jp

Received July 13, 2008; Accepted in revised form September 1, 2008.

Introduction

Angiogenesis has opposite effects, depending on disease conditions (*1*). In ischemic vascular disorders such as peripheral artery disease and ischemic heart disease, therapeutic angiogenesis provides beneficial effects and is recognized as useful for the treatment of such disorders (*2*). Recently, hepatocyte growth factor (HGF), which is well recognized to augment angiogenesis (*3*), has been employed in clinical applications for therapeutic angiogenesis (*4*). 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) have been reported to promote angiogenesis and ameliorate ischemic conditions (*5*, *6*). Statins may therefore be a potential adjuvant for HGF in terms of therapeutic angiogenesis. However, their effects on the HGF-induced angiogenesis remain unclear.

In the present study, we examined the effects of fluvastatin on HGF-induced angiogenesis using a human umbilical vein endothelial cell (EC) (HUVEC)/normal human dermal fibroblast (NHDF) co-culture system (*7*, *8*). Fluvastatin at low dose, but not at high dose, augmented the HGF-induced angiogenesis. Our data also indicated that the stimulatory effects of low-dose fluvastatin on the HGF-induced angiogenesis were mediated by the suppression of the p38 mitogen-activated protein kinase (MAPK) pathway.

Methods

Reagents

Human recombinant HGF was purchased from BD Biosciences (Bedford, USA). Fluvastatin was provided by Novartis Pharma (Tokyo, Japan). FR167653 was provided by Astellas Pharma (Tokyo, Japan). SB202474, SB203580, and SB202190 were purchased from Calbiochem (San Diego, USA). Polyclonal anti-phospho p38 MAPK (Thr-180/Tyr-182) and anti-p38 MAPK, anti-phospho Akt (Ser-473), and anti-Akt antibodies were purchased from Cell Signaling Technology (Danvers, USA). LY294002 and anisomycin were purchased from Sigma-Aldrich (St. Louis, USA).

Cell Culture

HUVECs and NHDFs were purchased from Kurabo (Osaka, Japan). They were maintained in HuMedia-EB2 (Kurabo) supplemented with 2% fetal bovine serum (FBS), 10 ng/mL epithelial growth factor (EGF), 5 ng/mL fibroblast growth factor (FGF)-2, 1 μg/mL hydrocortisone, 10 μg/mL heparin, 50 μg/mL gentamicin and 50 ng/mL amphotericin-B. NHDFs were maintained in M-106S (Cascade Biologics, Portland, USA) supplemented with 2% FBS, 10 ng/mL EGF, 3 ng/mL FGF-2, 1 μg/mL hydrocortisone, 10 μg/mL heparin, 100 μg/mL streptomycin and 100 μg/mL penicillin. These cells were cultured in a humidified incubator under 5% CO_2 at 37°C and used between the 2nd and the 5th passages.

Angiogenesis

Angiogenesis assays were performed using the Angiogenesis kit (Kurabo) (*7*, *8*), according to the manufacturer's instructions. Briefly, HUVECs and NHDFs were co-cultured in media as monolayers in plastic 24-multiwell plates for 11 d. HGF, fluvastatin, or other reagents were added to the media, which were refreshed at days 1, 4, 7, and 9. After the incubation periods, tube structures were stained by anti-human CD31 antibody and 3,3-diamino benzidine tetrahydrochloride (DAB) substrate using a tubule staining kit (Kurabo) according to the manufacturer's instructions. We determined tube formation quantitatively by the total length of the tube structure using the Angiogenesis Image Analyzer software (Kurabo).

RNA Isolation and Real-Time PCR

When cells grown in 6-multiwell plates became 80% confluent, the media were changed to DMEM supplemented with 2% FBS. The cells were treated with or without fluvastatin for 24 h, and total RNA was extracted as previously described (*9*) with the use of the RNeasy Plus Mini kit (QIAGEN, Hilden, Germany). The extracted RNA was used for reverse transcription (RT)-reaction by RNA PCR Kit (AMV), version 3.0 (Takara Bio, Ohtsu, Japan), using random 9-mer primers. RT reaction was performed under the following conditions: 30°C, 10 min, 42°C, 30 min and 99°C, 5 min. For integrins and GAPDH, the obtained templates (100 ng) were used for real-time PCR reactions (95°C, 3 min for 1 cycle; 95°C, 15 s; 60°C, 10 s; 72°C, 20 s; for 40 cycles) with the use of iQ SYBR green Supermix (Bio-Rad, Hercules, USA) by DNA Engine thermal cycler attached with Chromo4 detector (Bio-Rad). The following primer sequences were used: $p27^{kip1}$ (forward: 5′-tttgacttgcatgaagagaagc-3′; reverse: 5′-agctgtctc tgaaagggacatt-3′; PCR product: 84 bp), integrin α2 (forward: 5′-caagtgggattcagtgcaga-3′; reverse: 5′-gagcaccagcaacaaagt ga-3′; PCR product: 232 bp), integrin α5 (forward: 5′-agcctc agaaggaggaggac-3′; reverse: 5′-ggttaatggggtgattggtg-3′; PCR product: 186 bp), integrin α6 (forward: 5′-tttatcggtctcggg agttg-3′; reverse: 5′-tggccactgaatgttcaagg-3′; PCR product: 181 bp), integrin αV (forward: 5′-caccagcagtcagagatgga-3′; reverse: 5′-acaactggcccaacatcttc-3′; PCR product: 224 bp), integrin β1 (forward: 5′-cgatgccatcatgcaagt-3′; reverse: 5′-acaccagcagccgtgtaac-3′; PCR product: 71 bp), integrin β3 (forward: 5′-gtgtgcctggtgctctgat-3′; reverse: 5′-agcagattctcc ttcaggtca-3′; PCR product: 69 bp), GAPDH (forward: 5′-ccatggagaaggctgggg-3′; reverse: 5′-caaagttgtcatggatgacc-3′; PCR product: 195 bp). For other genes, real-time PCR was performed with LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Basel, Switzerland) and LightCycler Primer Sets for vascular endothelial growth factor (VEGF), matrix metalloproteinase (MMP)-2, tissue inhibitor

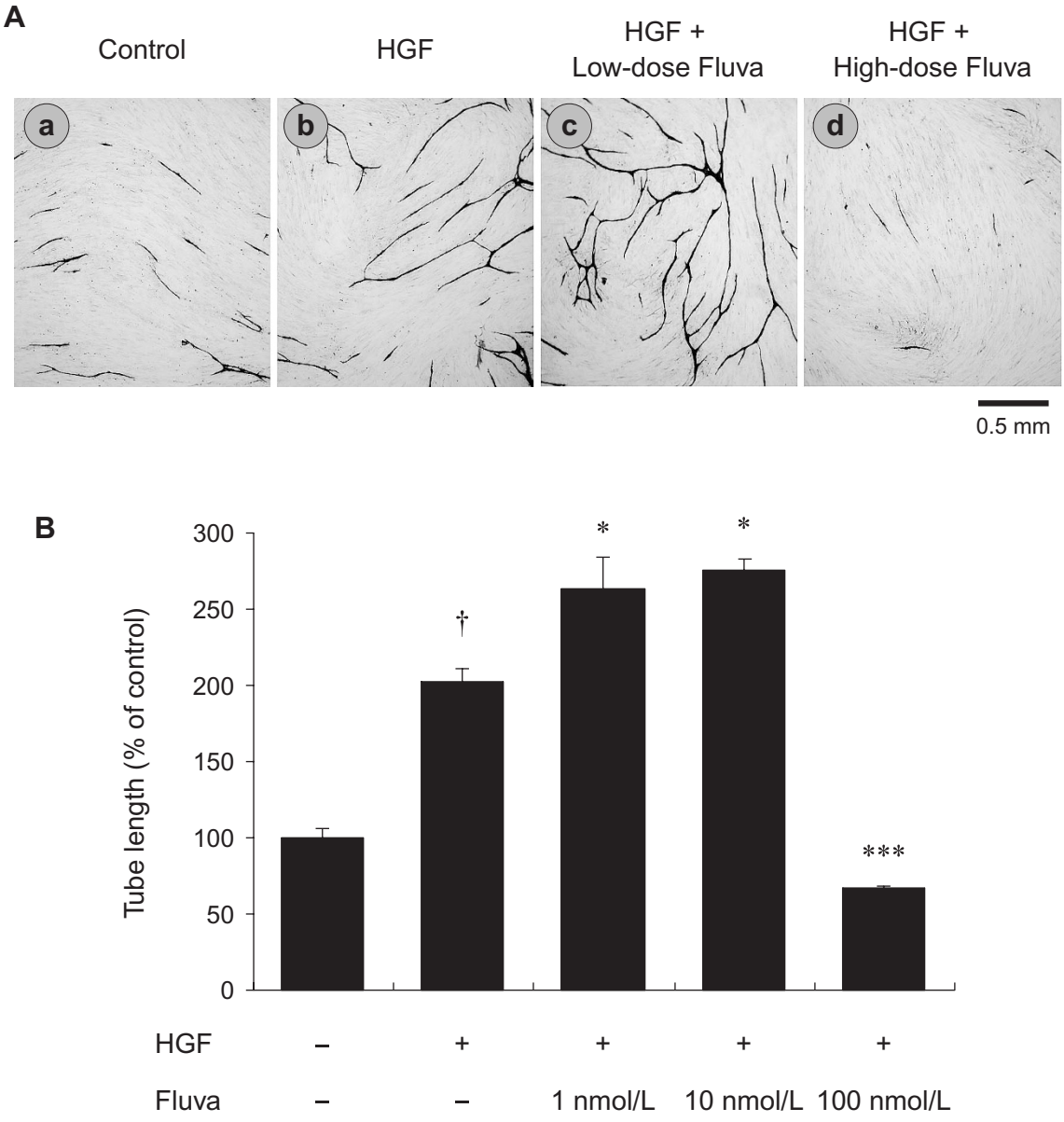


Fig. 1. *Effects of fluvastatin on angiogenesis in an HUVEC/NHDF co-culture system. A: Microphotographs of angiogenesis. The cells were incubated in either the absence (a) or presence (b, c, d) of HGF at 30 ng/mL, without (a, b) or with fluvastatin at (c) 3 or (d) 100 nmol/L for 11 d. B: Graph of quantified tube length, percent of control.* $^{\dagger}p < 0.01$ *vs. control.* $^{*}p < 0.05$, $^{***}p < 0.001$ *vs. HGF alone. Fluva, fluvastatin.*

of metalloproteinase (TIMP)-1, and TIMP-2 (Search LC, Heidelberg, Germany).

Measurement of VEGF Concentration

When NHDFs grown in 6-multiwell plates became 80% confluent, the media were changed to 1 mL/well of 2% FBS–containing DMEM either with or without fluvastatin. The cells were thereafter incubated for 24 h. VEGF concentrations of media were measured by enzyme-linked immunosorbent assay (ELISA) with the Biotrak Vascular Endothelial Growth Factor Human ELISA System (GE Healthcare, Buckinghamshire, UK). The assay range of VEGF was 31.3 to 2,000 pg/mL. The VEGF concentrations of 2% FBS–containing media without cells were below the lower limit of detection. All experiments were performed in duplicate for each sample.

Protein Extraction and Western Blotting

When HUVECs grown in 6-multiwell plates became 80%

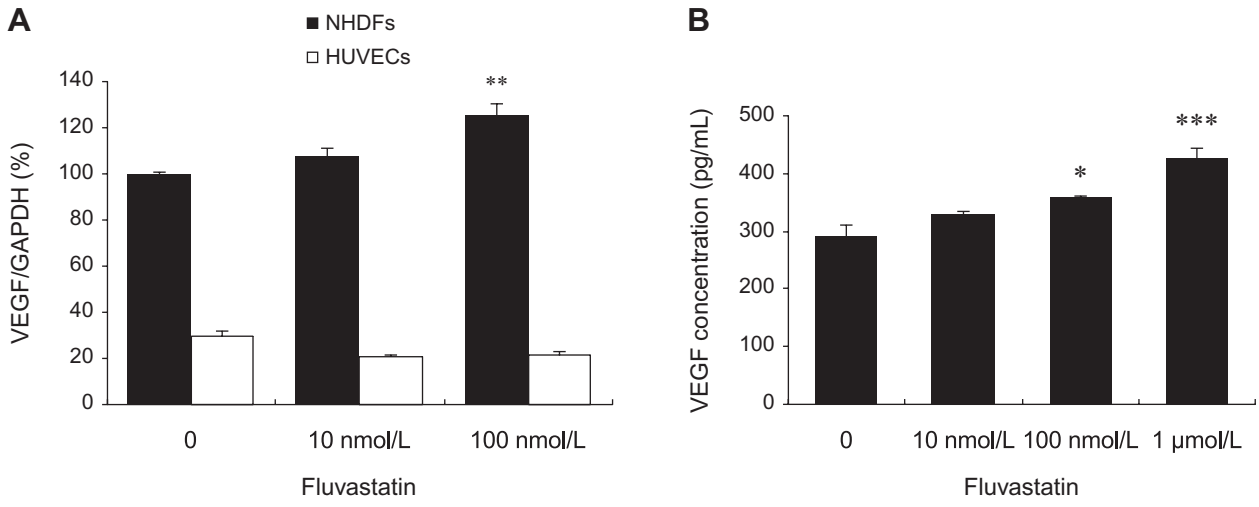


Fig. 2. *Effects of fluvastatin on VEGF expression. A: VEGF mRNA expression either in NHDFs (filled bars) or in HUVECs (open bars) determined by real-time PCR analyses. NHDFs or HUVECs were cultured and incubated in 2% FBS–containing DMEM with or without fluvastatin at indicated concentrations for 24 h. Data are shown as percent of NHDFs in the absence of fluvastatin, VEGF mRNA levels normalized by GAPDH mRNA levels. **$p < 0.01$ vs. control. B: VEGF concentrations in NHDFs mono-cultured media determined by ELISA. Data are shown as pg/mL. *$p < 0.05$, ***$p < 0.001$ vs. control.*

confluent, the media were changed to DMEM supplemented with 2% FBS. The cells were treated with or without fluvastatin for 24 h. HUVECs were rinsed twice with PBS. Total cellular extracts were prepared and denatured as previously described (*10*, *11*). The protein samples were separated by SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Bio-Rad). The membranes were blocked with 5% nonfat dry milk for 1 h at room temperature and then probed with primary antibodies and horseradish peroxidase–conjugated secondary antibodies (GE Healthcare). The membranes were visualized with ECL-plus (GE Healthcare) and LAS-1000 (Fujifilm, Tokyo, Japan).

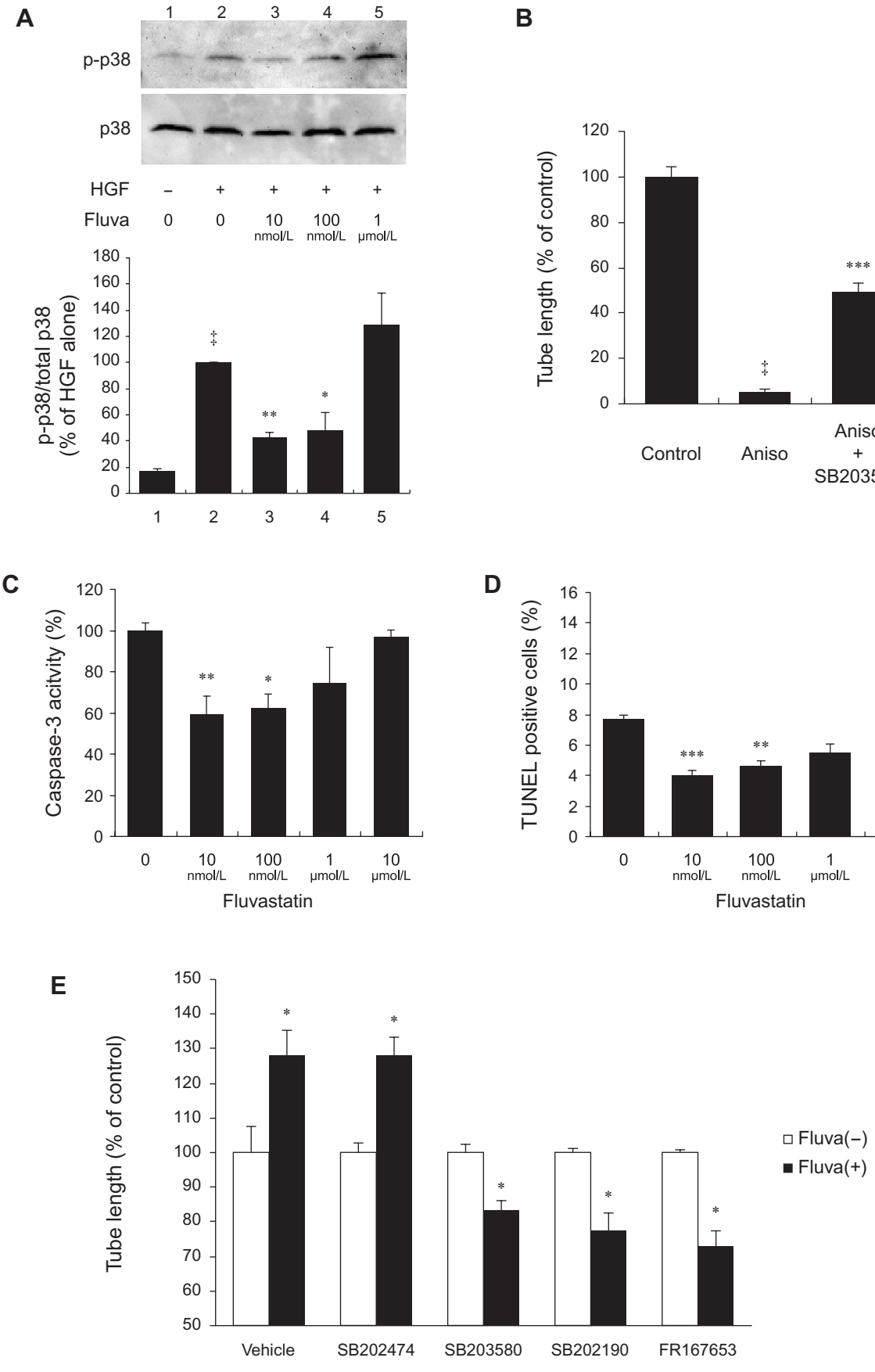
Measurement of Caspase-3 Activity

When HUVECs grown in 6-multiwell plates became 80% confluent, cells were washed once with PBS, and media were changed to DMEM supplemented with 0.5% FBS and HGF (30 ng/mL). The cells were treated with or without fluvastatin for 24 h. A caspase-3 activity assay was performed with an APOCYTO Caspase-3 Colorimetric Assay Kit (Medical Biological Laboratories, Nagoya, Japan), according to the manufacturer's instructions.

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End–Labeling Staining

When HUVECs grown in 24-multiwell plates became 80%

Fig. 3. *Effects of fluvastatin on p38 MAPK phosphorylation/EC apoptosis. A: Effects of fluvastatin on p38 MAPK phosphorylation. HUVECs were incubated in 2% FBS–containing DMEM without (lanes 1 and 2) or with (lanes 3–5) varying concentrations of fluvastatin for 24 h. The cells were exposed to 30 ng/mL HGF (for 10 min, lanes 2–5). Aliquots of cell lysates (25 μg each) were used for Western immunoblot analyses using antibodies raised against either phospholylated-p38 MAPK at Thr-180/Tyr-182 (dilution 1:500, upper) or p38 MAPK (1:1,000, lower). Data are shown as densitometric units of each phosphorylated protein normalized with each total p38 MAPK. ‡$p < 0.001$, vs. lane 1. *$p < 0.05$, **$p < 0.01$ vs. lane 2. B: Effects of anisomycin on angiogenesis. Cells incubated in the presence of HGF (30 ng/mL) were treated without or with anisomycin (1 μmol/L) in the presence or absence of SB203580 (10 μmol/L). C: Caspase-3 activity in HUVECs. Cells incubated with DMEM supplemented with 0.5% FBS and HGF (30 ng/mL) were treated with or without fluvastatin for 24 h. *$p < 0.05$, **$p < 0.01$ vs. without fluvastatin. D: HUVEC apoptosis determined by TUNEL staining. The cells were incubated with DMEM supplemented with 0.5% FBS and HGF (30 ng/mL), treated with or without fluvastatin for 24 h. Data are shown as percent of TUNEL-positive HUVECs. **$p < 0.01$, ***$p < 0.001$ vs. without fluvastatin. E: Effects of p38 MAPK inhibitors on angiogenesis. The cells were incubated in the presence of HGF (30 ng/mL) without (open bars) or with (filled bars) fluvastatin at 3 nmol/L for 11 d and were co-administered with the indicated compounds (0.1% DMSO, 10 μmol/L SB202474, 10 μmol/L SB203580, 10 μmol/L SB202190, 10 μmol/L FR167653). Data are shown as percentage of fluva(−) group tube length. *$p < 0.05$ vs. fluva(−). Fluva, fluvastatin; p-, phosphorylated-.*


A
1 2 3 4 5
p-p38
p38
HGF – + + + +
Fluva 0 0 10 nmol/L 100 nmol/L 1 μmol/L
p-p38/total p38 (% of HGF alone)
B
Tube length (% of control)
Control
Aniso
Aniso + SB203580
C
Caspase-3 acitvity (%)
Fluvastatin
D
TUNEL positive cells (%)
Fluvastatin
E
Tube length (% of control)
Fluva(–)
Fluva(+)
Vehicle
SB202474
SB203580
SB202190
FR167653

confluent, the media were changed to DMEM supplemented with 0.5% FBS and HGF (30 ng/mL). The cells were treated with or without fluvastatin for 24 h. Terminal deoxynucleotidyl transferase–mediated dUTP nick end–labeling (TUNEL) staining was performed with the *In Situ* Cell Death Detection Kit (Roche Diagnostics), according to the manufacturer's instructions. Color reactions were developed with DAB peroxidase. TUNEL-positive cells per total cells counted were determined. At least 200 cells were counted.

Cell Proliferation Assay by 5-Bromo-2′-Deoxy-Uridine Incorporation

Cell proliferation was determined by 5-bromo-2′-deoxy-uridine (BrdU) incorporation. HUVECs were seeded in 24-multiwell plates. After incubation with maintenance media for 24 h, the media were changed to DMEM supplemented with 1% FBS and HGF (30 ng/mL). The cells were treated with or without fluvastatin for 24, 48, or 72 h. For the last 12 h of the incubation periods, BrdU was added to the media, and BrdU incorporation was measured using the Cell Proliferation ELISA, BrdU colorimetric kit (Roche Diagnostics), according to the manufacturer's instructions.

Statistical Analysis

Data are presented as mean±SEM. Statistical comparisons were performed using ANOVA followed by Fisher's LSD post hoc test. $p<0.05$ was considered significant.

Results

Fluvastatin Regulates the HGF-Induced Angiogenesis in HUVEC/NHDF Co-Culture System

To determine the effects of fluvastatin on the HGF-induced angiogenesis, we performed an angiogenesis assay using a HUVEC/NHDF co-culture system. As shown in Fig. 1A, HGF increased angiogenesis (b), and the HGF-induced angiogenesis was augmented by low-dose fluvastatin (c) but suppressed by high-dose fluvastatin (d). Figure 1B shows the tube lengths in the HUVEC/NHDF co-culture system. HGF significantly increased the tube lengths (202.5±8.4% of control). Fluvastatin significantly augmented the HGF-induced angiogenesis at 1 and 10 nmol/L (264±20.1% and 275.7±7.2% of control, respectively) but suppressed it at 100 nmol/L (66.6±1.9% of control). These data indicate that low-dose, but not high-dose, fluvastatin augmented the HGF-induced angiogenesis.

High-Dose Fluvastatin Increases VEGF Expression in NHDFs

Next, the regulation of VEGF expression in NHDFs was examined. As shown in Fig. 2A, fluvastatin increased VEGF mRNA expression above 100 nmol/L in NHDFs (filled bars), but not in HUVECs (open bars). Fluvastatin also induced VEGF secretion from NHDFs above 100 nmol/L (Fig. 2B). These data indicate that high-dose, but not low-dose, fluvastatin increased NHDF VEGF mRNA expression/secretion.

Low-Dose Fluvastatin Decreases p38 MAPK Phosphorylation and HUVEC Apoptosis

To explore the mechanisms of stimulatory effects of low-dose fluvastatin on the HGF-induced angiogenesis, we focused on p38 MAPK because of its anti-angiogenic properties (*12*). As shown in Fig. 3A, HGF induced p38 MAPK phosphorylation in HUVECs (lane 2). The HGF-induced p38 MAPK phosphorylation was suppressed by fluvastatin at 10–100 nmol/L (lanes 3 and 4 showed 42.8±3.9% and 47.9±13.7% of HGF alone, respectively), but not at 1 μmol/L (lane 5). Next, we examined the effects of p38 MAPK on the HGF-induced angiogenesis in the HUVEC/NHDF co-culture system. As shown in Fig. 3B, anisomycin, an inducer of p38 MAPK, strongly inhibited angiogenesis in the presence of HGF (5.2±1.4% of control), and SB203580, an inhibitor of p38 MAPK, reduced the inhibitory effect of anisomycin (49.1±4.1% of control), suggesting that p38 MAPK acts as an angiogenesis suppressor in the system. Since p38 MAPK induces apoptosis in ECs (*13*) and EC apoptosis inhibits angiogenesis (*14*), we evaluated the effects of fluvastatin on EC apoptosis by measurement of caspase-3 activity and TUNEL staining. As shown in Fig. 3C, fluvastatin decreased caspase-3 activity at 10–100 nmol/L, but not at above 1 μmol/L. Moreover, the number of TUNEL-positive HUVECs was decreased by fluvastatin at 10–100 nmol/L, while it increased at 10 μmol/L (Fig. 3D). These data suggest that low-dose fluvastatin inhibited the p38 MAPK activity, which may have resulted in a decrease in EC apoptosis.

Fluvastatin Does Not Increase Angiogenesis in the Presence of p38 MAPK Inhibitors

Next, we examined whether fluvastatin can increase angiogenesis in the presence of p38 MAPK inhibitors. As shown in Fig. 3E, the tube length in the fluvastatin(+) group (closed bars) was greater than that in the fluvastatin(−) group (open bars) when treated with either the vehicle (128.1±7.2% of fluvastatin(−) group) or an inactive compound, SB202474 (128.2±5.1% of fluvastatin(−) group). However, treatment with p38 MAPK inhibitors SB203580, SB202190, or FR167653 significantly lowered the tube length in the fluvastatin(+) group compared with that in the fluvastatin(−) group (83.2±2.8%, 77.4±5.1%, 72.9±4.4% of fluvastatin(−) group, respectively). These data therefore suggest that p38 MAPK was important for the increase in angiogenesis caused by fluvastatin.

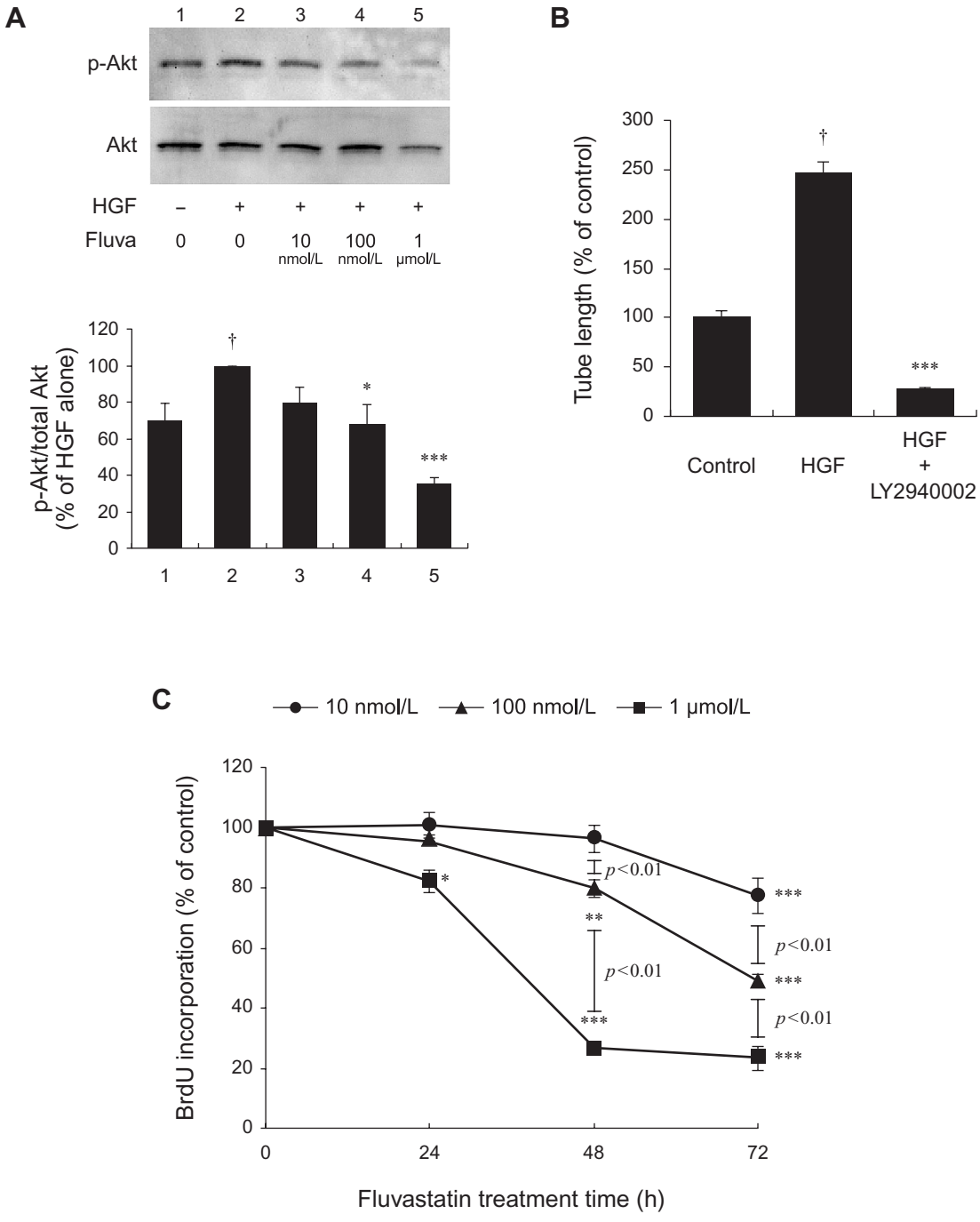


Fig. 4. *Effects of fluvastatin on Akt phosphorylation/EC proliferation. A: Effects of fluvastatin on Akt phosphorylation. HUVECs were incubated in 2% FBS–containing DMEM without (lanes 1 and 2) or with (lanes 3–5) varying concentrations of fluvastatin for 24 h. The cells were exposed to 30 ng/mL HGF (for 24 h, lanes 2–5). Aliquots of cell lysates (10 μg each) were used for Western immunoblot analyses using antibody raised against either phosphorylated-Akt at Ser-473 (dilution 1:1,000, upper) or Akt (1:1,000, lower). Data are shown as densitometric units of each phosphorylated protein normalized with each total p38 MAPK. †$p<0.05$, vs. lane 1. *$p<0.05$, ***$p<0.001$ vs. lane 2. B: Effects of LY294002 on angiogenesis. The cells were incubated without or with HGF (30 ng/mL) in the presence or absence of LY294002 (10 μmol/L) for 11 d. †$p<0.001$, vs. lane 1. ***$p<0.001$ vs. HGF alone. C: Effects of fluvastatin on EC proliferation. Cell proliferation was determined by BrdU incorporation. HUVECs incubated with DMEM supplemented with 1% FBS and HGF (30 ng/mL) were treated with or without fluvastatin (closed circle: 10 nmol/L; closed triangle: 100 nmol/L; closed box: 1 μmol/L) for indicated times. Data are shown as percent of the level without fluvastatin. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. without fluvastatin. Fluva, fluvastatin; p-, phosphorylated-.*

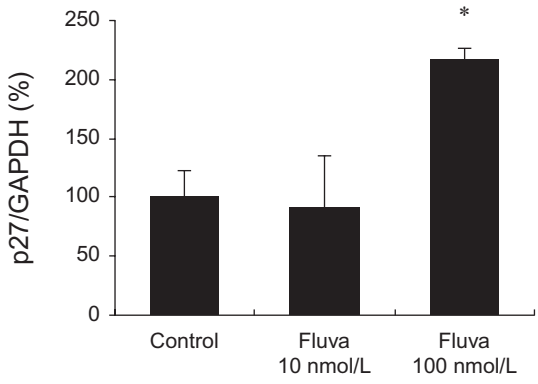


Fig. 5. *Effect of fluvastatin on p27^{kip1} mRNA expression in HUVECs determined by real-time PCR analyses. HUVECs were cultured and incubated in 2% FBS–containing DMEM with or without fluvastatin at indicated concentrations for 24 h. Data are shown as percent of the levels without fluvastatin. p27^{kip1} mRNA levels were normalized by GAPDH mRNA levels. *$p < 0.05$ vs. control.*

High-Dose Fluvastatin Suppresses Phosphorylation of Akt and HUVEC Proliferation

Fluvastatin at 100 nmol/L decreased p38 MAPK phosphorylation and HUVEC apoptosis (Fig. 3), while it decreased the HGF-induced angiogenesis in the HUVEC/NHDF system (Fig. 1). Therefore, we focused on phosphoinositide 3-kinase (PI3K)–Akt pathway (*15*) and HUVEC proliferation. HGF increased Akt phosphorylation in HUVECs (Fig. 4A, lane 2). However, the HGF-induced Akt phosphorylation was suppressed by fluvastatin at 100 nmol/L and 1 μmol/L (lanes 4 and 5 showed 67.6±11.0% and 35.0±3.7% of HGF alone, respectively). As shown in Fig. 4B, HGF increased angiogenesis (247.0±10.5% of control), and LY294002, an inhibitor of PI3K, strongly suppressed the HGF-induced angiogenesis (27.6±1.2% of control) in the HUVEC/NHDF system. To determine HUVEC proliferation, we performed a BrdU incorporation assay. Fluvastatin decreased BrdU incorporation in the HUVECs in a dose- and time-dependent manner (Fig. 4C). These data indicate that high-dose fluvastatin inhibits Akt activity as well as EC proliferation.

High-Dose Fluvastatin Increases mRNA Expression of p27^{kip1}

We then examined whether fluvastatin affects expression of 27^{kip1}, a cyclin-dependent kinase inhibitor, in HUVECs. As shown in Fig. 5, fluvastatin increased 27^{kip1} mRNA expression at 100 nmol/L (216.2±10.6% of control). However, fluvastatin at 10 nmol/L did not affect 27^{kip1} mRNA expression.

Fluvastatin Decreases mRNA Expression of Integrins, TIMPs, and MMP-2

To explore candidates other than those in the p38 MAPK and PI3K-Akt pathways that may be regulated by fluvastatin during angiogenesis, we examined the mRNA expression of integrins in HUVECs by real-time PCR (*16*). As shown in Fig. 6, fluvastatin decreased the mRNA expression of integrin α2, α5, αV, β1, and β3 at 10 nmol/L (75.2±5.3%, 63.8±1.8%, 66.8±6.2%, 60.8±3.1%, and 72.8±4.6% of control, respectively) and also decreased that of integrin α2, α5, β1, and β3 at 100 nmol/L (83.4±5.3%, 76.3±4.4%, 71.0±7.8%, and 80.2±5.9% of control, respectively). Next, we examined the mRNA expression of TIMPs (*17*) and MMPs (*18*) in HUVECs and NHDFs by real-time PCR. As shown in Fig. 7, fluvastatin decreased TIMP-1 and -2 mRNA expression at 10 nmol/L in HUVECs (55.7±5.0% and 57.2±5.6% of control, respectively), but not at 100 nmol/L. Fluvastatin decreased the mRNA expression of MMP-2 at 10 and 100 nmol/L in HUVECs (69.2±2.4% and 72.3±8.9% of control, respectively) as well as at 100 nmol/L in NHDFs (79.4±3.4% of control). These data therefore raise the possibility that these integrins, TIMPs, and MMP-2 may be involved in the fluvastatin effect.

Discussion

Therapeutic angiogenesis using HGF has recently been applied in the clinical treatment of ischemic vascular disorders (*2*). In the present study, we first demonstrated that fluvastatin at low dose, but not at high dose, augmented the HGF-induced angiogenesis (Fig. 1). Although the pro-angiogenic effects of low-dose statins *in vitro* have been previously reported (*19*, *20*), our study clearly demonstrated that low-dose fluvastatin might also be effective as an adjuvant for HGF for therapeutic angiogenesis. The mechanisms of the up-regulation of angiogenesis by statins have not been adequately elucidated so far. The phosphorylation of Akt (*21*) and endothelial nitric oxide synthase (*22*) by statins has been reported to be involved in their pro-angiogenic effects. Also, statins have been reported to increase VEGF production in HUVECs (*23*). Although we also observed statin-induced increase of VEGF expression in NHDFs (Fig. 2), it occurred only with high-dose fluvastatin that actually decreased the HGF-induced angiogenesis in the HUVEC/NHDF co-culture system (Fig. 1). Since the vascular effects of statins are quite complicated (*24*), it is possible that other mechanisms may also be involved in the up-regulation of angiogenesis by low-dose fluvastatin, especially in the presence of HGF.

The protein p38 MAPK is activated by the tumor necrosis factor-α and other stresses *via* its dual phosphorylation of threonine and tyrosine residues (*25*), and it has been reported to be anti-angiogeneic (*12*). Moreover, it has also been reported to induce apoptosis in ECs (*13*). On the other hand, statins have been shown to decrease p38 MAPK phosphoryla-

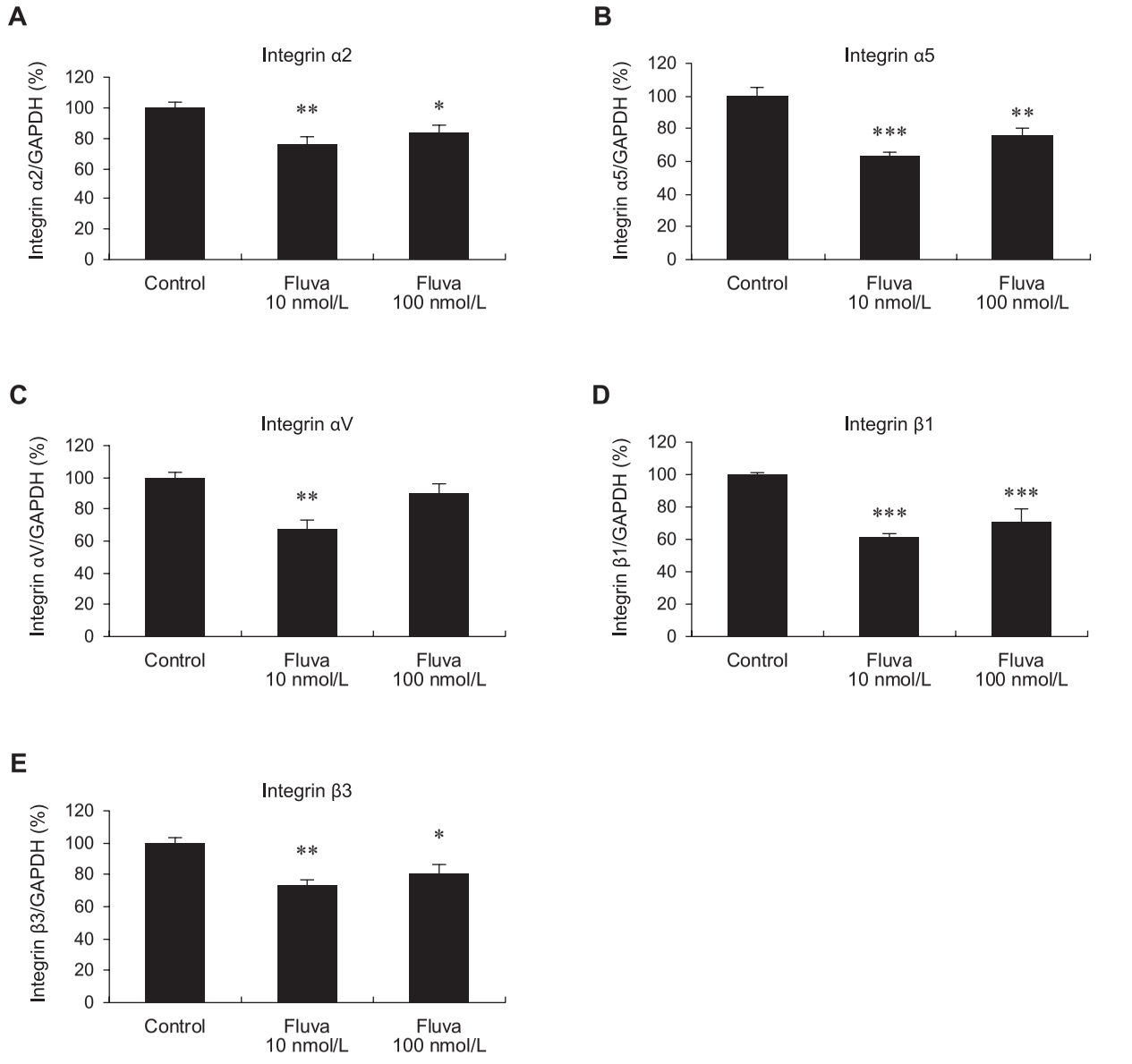


Fig. 6. *Effects of fluvastatin on integrins mRNA expression. Integrin α2 (A), α5 (B), αV (C), β1 (D), and β3 (E) mRNA expression in HUVECs was determined by real-time PCR analyses. HUVECs were cultured and incubated in 2% FBS–containing DMEM with or without fluvastatin at indicated concentrations for 24 h. Data are shown as percent of the levels without fluvastatin. Integrins mRNA levels were normalized by GAPDH mRNA levels. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. control. Fluva, fluvastatin.*

tion in several cells/tissues including vascular smooth muscle cells (*26*), myocardium (*27*), and macrophages (*28*). In the present study, we demonstrated that 1) low-dose fluvastatin decreased apoptosis in HUVECs in the presence of HGF, 2) low-dose fluvastatin suppressed p38 MAPK phosphorylation in HUVECs in the presence of HGF, and 3) SB203580 reduced the anisomycin-induced angiogenesis suppression in the presence of HGF (Fig. 3). Also, HGF was demonstrated to activate (phosphorylate) p38 MAPK in HUVECs (Fig. 3A). Therefore, although HGF is a potent inducer of angiogenesis, it also activates p38 MAPK which is a negative regulator of angiogenesis. Taken together, the stimulatory effects of low-dose fluvastatin on the HGF-induced angiogenesis may be due to its inhibitory effects on p38 MAPK phosphorylation induced by HGF, which may result in the suppression of EC apoptosis. Consistent with this notion, low-dose fluvastatin could not augment the HGF-induced angiogenesis in the presence of p38 MAPK inhibitors (Fig. 3E), indicating that the

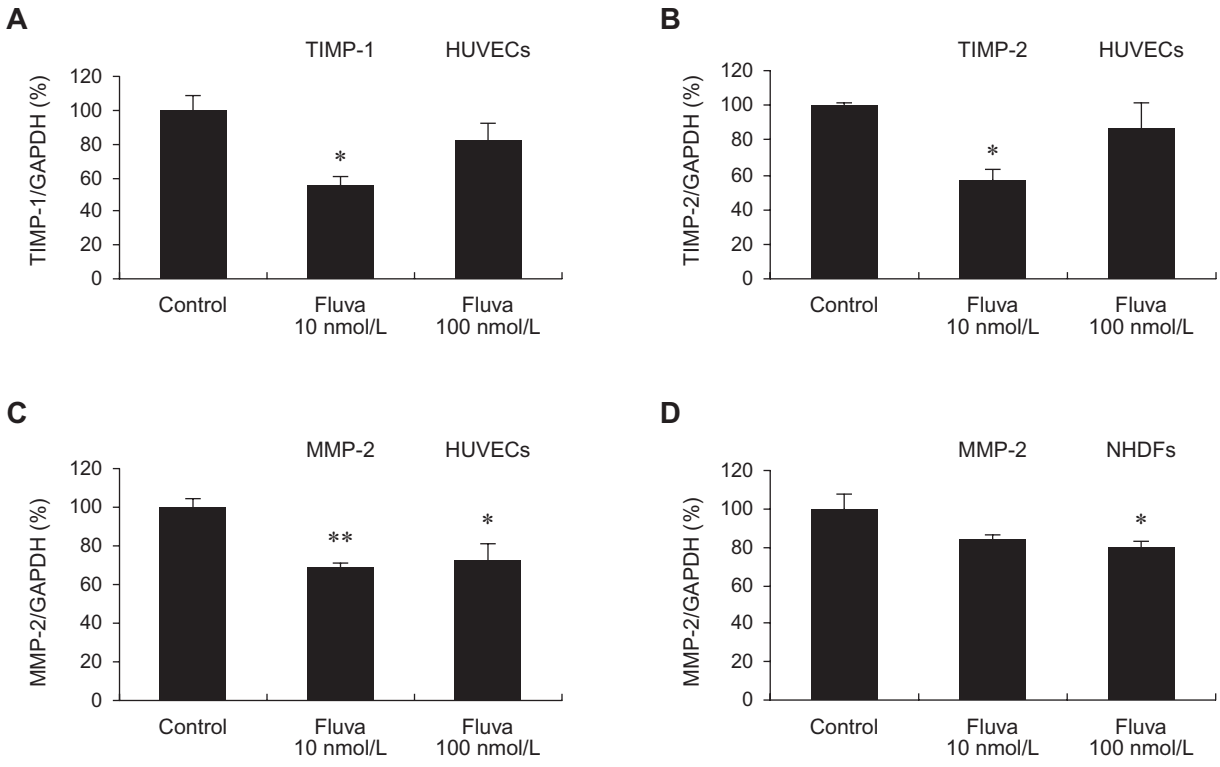


Fig. 7. *Effects of fluvastatin on TIMPs and MMP-2 mRNA expression. TIMP-1 in HUVECs (A), TIMP-2 in HUVECs (B), MMP-2 in HUVECs (C), and MMP-2 in NHDFs (D) mRNA expression was determined by real-time PCR analyses. HUVECs or NHDFs were cultured and incubated in 2% FBS–containing DMEM with or without fluvastatin at indicated concentrations for 24 h. Data are shown as percent of the levels without fluvastatin. TIMPs or MMP-2 mRNA levels were normalized by GAPDH mRNA levels. *$p < 0.05$, **$p < 0.01$ vs. control. Fluva, fluvastatin.*

presence of p38 MAPK phosphorylation is essential for these effects. The inhibition of p38 MAPK has been shown to exert some favorable effects on the vasculature, including the prevention of neointimal formation after balloon injury (*29*) and the induction of antioxidant reactions in the presence of hypertension (*30*). Therefore, the inhibitory effects of low-dose fluvastatin on p38 MAPK phosphorylation may not only augment angiogenesis but also provide vascular protective effects.

High-dose fluvastatin decreased the HGF-induced angiogenesis (Fig. 1), Akt phosphorylation (Fig. 4A), and HUVEC proliferation (Fig. 4C). The HGF-induced angiogenesis was suppressed by LY294002, a PI3K inhibitor, in the HUVEC/NHDF co-culture system. Therefore, the inhibitory effects of high-dose fluvastatin on the HGF-induced Akt phosphorylation and HUVEC proliferation may potentially contribute to the suppression of angiogenesis (*14*).

Regarding HUVEC proliferation, fluvastatin at 100 nmol/L decreased the proliferation significantly compared with fluvastatin at 10 nmol/L (Fig. 4C). Although fluvastatin at 100 nmol/L significantly decreased the HGF-induced Akt phosphorylation level, this level was not significantly different from that observed with fluvastatin at 10 nmol/L (Fig. 4A). Therefore, other mechanism(s) may also contribute to the high-dose fluvastatin-mediated suppression of HUVEC proliferation. Because fluvastatin has been reported to affect cell cycles *via* an increase in the expression of the cyclin-dependent kinase inhibitor $p27^{kip1}$ in kidney fibroblasts (*31*), prostate cancer cell lines (*32*), and smooth muscle cells (*33*), and because the cyclin-dependent kinase inhibitor has been shown to regulate EC cell cycles (*34*), we examined $p27^{kip1}$ expression in HUVECs. Interestingly, fluvastatin at 100 nmol/L, but not at 10 nmol/L, increased $p27^{kip1}$ expression (Fig. 5). Therefore, the increase of $p27^{kip1}$ expression by fluvastatin may possibly account for the suppression of HUVEC proliferation with high-dose fluvastatin.

In the present study, fluvastatin at 100 nmol/L induced a 1.2-fold increase in the VEGF concentration of the NHDFs culture media (Fig. 2B, from 299±20 to 359±2 pg/mL), despite its strong suppression of the HGF-induced angiogenesis *in vitro* (Fig. 1). Since the effects of VEGF on HUVEC proliferation did not vary between 300 and 500 pg/mL of its concentration in the previous study (*35*), the 1.2-fold increase in VEGF concentration may not be sufficient to maintain HUVEC proliferation in the presence of high-dose fluvastatin. The HGF concentration (30 ng/mL) used in the Akt

phosphorylation experiment was approximately 100-fold higher than the VEGF concentration (Fig. 2B, 359±2 pg/mL) in the NHDFs culture media incubated with 100 nmol/L fluvastatin. It is therefore likely that the inhibitory effects of 100 nmol/L fluvastatin on the HGF-induced Akt phosphorylation observed in HUVECs (Fig. 4A) can also be observed in the HUVEC/NHDF co-culture system, since the amount of VEGF secreted from NHDFs is negligible. The notion is also supported by a previous report that demonstrated that 50–500 nmol/L statin (simvastatin) inhibited 10 ng/mL VEGF-induced Akt phosphorylation in ECs (*36*).

Integrins constitute the extracellular matrix. Because they regulate angiogenesis, they are potential therapeutic targets for tumor angiogenesis (*14*, *37*, *38*). Fluvastatin decreased the mRNA expression of integrins in HUVECs (Fig. 6). Integrins have pleiotropic effects in EC function and angiogenesis when there is contact between ECs and interstitial collagens or fibrin matrices; such contact leads to EC activation/morphogenesis as well as EC differentiation/stabilization when the basement membrane matrices around the developing tubes accumulate (*16*). Further studies are needed to elucidate the effects of integrins on fluvastatin-mediated angiogenesis regulation. The significant decrease in angiogenesis by low-dose fluvastatin in the presence of p38 MAPK inhibitors (Fig. 3E) may be due partly to the observed decrease in integrins mRNA expression by low-dose fluvastatin in HUVECs (Fig. 6). In addition, fluvastatin also decreased TIMPs and MMP-2 (Fig. 7). MMPs promote vascular remodeling and angiogenesis, and TIMPs are endogenous inhibitors of MMPs (*39*), which increase in concentration in hypertensive patients (*40*). Low-dose fluvastatin decreased TIMP-1 and -2 mRNA expression. Moreover, fluvastatin also decreased MMP-2 mRNA expression in HUVECs and NHDFs. These results therefore indicate that the suppression of TIMPs may partially contribute to the stimulatory angiogenesis regulation by low-dose fluvastatin.

In conclusion, our findings suggest that low-dose fluvastatin augments the HGF-induced angiogenesis. This may occur through the suppression of EC apoptosis subsequent to the HGF-induced inhibition of p38 MAPK. High-dose fluvastatin may inhibit angiogenesis through the suppression of Akt and EC proliferation and the increase of $p27^{kip1}$.

References

1. Walsh DA: Pathophysiological mechanisms of angiogenesis. *Adv Clin Chem* 2007; **44**: 187–221.
2. Takeshita S, Zheng LP, Brogi E, *et al*: Therapeutic angiogenesis. A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. *J Clin Invest* 1994; **93**: 662–670.
3. Grant DS, Kleinman HK, Goldberg ID, *et al*: Scatter factor induces blood vessel formation *in vivo*. *Proc Natl Acad Sci U S A* 1993; **90**: 1937–1941.
4. Morishita R, Aoki M, Hashiya N, *et al*: Safety evaluation of clinical gene therapy using hepatocyte growth factor to treat peripheral arterial disease. *Hypertension* 2004; **44**: 203–209.
5. Pourati I, Kimmelstiel C, Rand W, Karas RH: Statin use is associated with enhanced collateralization of severely diseased coronary arteries. *Am Heart J* 2003; **146**: 876–881.
6. Sata M, Nishimatsu H, Osuga J, *et al*: Statins augment collateral growth in response to ischemia but they do not promote cancer and atherosclerosis. *Hypertension* 2004; **43**: 1214–1220.
7. Bishop ET, Bell GT, Bloor S, Broom IJ, Hendry NF, Wheatley DN: An *in vitro* model of angiogenesis: basic features. *Angiogenesis* 1999; **3**: 335–344.
8. Saito A, Sugawara A, Uruno A, *et al*: All-*trans* retinoic acid induces *in vitro* angiogenesis *via* retinoic acid receptor: possible involvement of paracrine effects of endogenous vascular endothelial growth factor signaling. *Endocrinology* 2007; **148**: 1412–1423.
9. Uruno A, Sugawara A, Kudo M, *et al*: Transcription suppression of thromboxane receptor gene expression by retinoids in vascular smooth muscle cells. *Hypertens Res* 2003; **26**: 815–821.
10. Uruno A, Sugawara A, Kanatsuka H, *et al*: Hepatocyte growth factor stimulates nitric oxide production through endothelial nitric oxide synthase activation by the phosphoinositide 3-kinase/Akt pathway and possibly by mitogen-activated protein kinase kinase in vascular endothelial cells. *Hypertens Res* 2004; **27**: 887–895.
11. Uruno A, Sugawara A, Kanatsuka H, *et al*: Upregulation of nitric oxide production in vascular endothelial cells by all-*trans* retinoic acid through the phosphoinositide 3-kinase/Akt pathway. *Circulation* 2005; **112**: 727–736.
12. Matsumoto T, Turesson I, Book M, Gerwins P, Claesson-Welsh L: p38 MAP kinase negatively regulates endothelial cell survival, proliferation, and differentiation in FGF-2-stimulated angiogenesis. *J Cell Biol* 2002; **156**: 149–160.
13. Nakagami H, Morishita R, Yamamoto K, *et al*: Phosphorylation of p38 mitogen-activated protein kinase downstream of bax−caspase-3 pathway leads to cell death induced by high D-glucose in human endothelial cells. *Diabetes* 2001; **50**: 1472–1481.
14. Folkman J: Angiogenesis and apoptosis. *Semin Cancer Biol* 2003; **13**: 159–167.
15. Liu W, Ahmad SA, Reinmuth N, *et al*: Endothelial cell survival and apoptosis in the tumor vasculature. *Apoptosis* 2000; **5**: 323–328.
16. Davis GE, Senger DR: Endothelial extracellular matrix: biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. *Circ Res* 2005; **97**: 1093–1107.
17. Raffetto JD, Khalil RA: Matrix metalloproteinases and their inhibitors in vascular remodeling and vascular disease. *Biochem Pharmacol* 2008; **75**: 346–359.
18. van Hinsbergh VW, Engelse MA, Quax PH: Pericellular proteases in angiogenesis and vasculogenesis. *Arterioscler Thromb Vasc Biol* 2006; **26**: 716–728.
19. Weis M, Heeschen C, Glassford AJ, Cooke JP: Statins have biphasic effects on angiogenesis. *Circulation* 2002; **105**: 739–745.
20. Urbich C, Dernbach E, Zeiher AM, Dimmeler S: Double-edged role of statins in angiogenesis signaling. *Circ Res*

2002; **90**: 737–744.
21. 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* 2000; **6**: 1004–1010.
22. Brouet A, Sonveaux P, Dessy C, Moniotte S, Balligand JL, Feron O: Hsp90 and caveolin are key targets for the proangiogenic nitric oxide-mediated effects of statins. *Circ Res* 2001; **89**: 866–873.
23. Frick M, Dulak J, Cisowski J, *et al*: Statins differentially regulate vascular endothelial growth factor synthesis in endothelial and vascular smooth muscle cells. *Atherosclerosis* 2003; **170**: 229–236.
24. Wang CY, Liu PY, Liao JK: Pleiotropic effects of statin therapy: molecular mechanisms and clinical results. *Trends Mol Med* 2008; **14**: 37–44.
25. New L, Han J: The p38 MAP kinase pathway and its biological function. *Trends Cardiovasc Med* 1998; **8**: 220–228.
26. McGillicuddy FC, O'Toole D, Hickey JA, Gallagher WM, Dawson KA, Keenan AK: TGF-beta1-induced thrombospondin-1 expression through the p38 MAPK pathway is abolished by fluvastatin in human coronary artery smooth muscle cells. *Vascul Pharmacol* 2006; **44**: 469–475.
27. Siddiqui AJ, Gustafsson T, Fischer H, *et al*: Simvastatin enhances myocardial angiogenesis induced by vascular endothelial growth factor gene transfer. *J Mol Cell Cardiol* 2004; **37**: 1235–1244.
28. Senokuchi T, Matsumura T, Sakai M, *et al*: Statins suppress oxidized low density lipoprotein-induced macrophage proliferation by inactivation of the small G protein–p38 MAPK pathway. *J Biol Chem* 2005; **280**: 6627–6633.
29. Ohashi N, Matsumori A, Furukawa Y, *et al*: Role of p38 mitogen-activated protein kinase in neointimal hyperplasia after vascular injury. *Arterioscler Thromb Vasc Biol* 2000; **20**: 2521–2526.
30. Tojo A, Onozato ML, Kobayashi N, Goto A, Matsuoka H, Fujita T: Antioxidative effect of p38 mitogen-activated protein kinase inhibitor in the kidney of hypertensive rat. *J Hypertens* 2005; **23**: 165–174.
31. Ikeuchi H, Kuroiwa T, Yamashita S, *et al*: Fluvastatin reduces renal fibroblast proliferation and production of type III collagen: therapeutic implications for tubulointerstitial fibrosis. *Nephron Exp Nephrol* 2004; **97**: e115–e122.
32. Sivaprasad U, Abbas T, Dutta A: Differential efficacy of 3-hydroxy-3-methylglutaryl CoA reductase inhibitors on the cell cycle of prostate cancer cells. *Mol Cancer Ther* 2006; **5**: 2310–2316.
33. Ferri N, Granata A, Pirola C, *et al*: Fluvastatin synergistically improves the antiproliferative effect of everolimus on rat smooth muscle cells by altering p27Kip1/cyclin E expression. *Mol Pharmacol* 2008; **74**: 144–153.
34. Suzuki E, Nishimatsu H, Nagata D, *et al*: Constitutive activation of proto-oncogen protein p21 induces cell cycle arrest in the G1 phase in contact-inhibited vascular endothelial cells. *Hypertens Res* 2002; **25**: 773–778.
35. Soker S, Gollamudi-Payne S, Fidder H, Charmahelli H, Klagsbrun M: Inhibition of vascular endothelial growth factor (VEGF)–induced endothelial cell proliferation by a peptide corresponding to the exon 7–encoded domain of VEGF165. *J Biol Chem* 1997; **272**: 31582–31588.
36. Park HJ, Kong D, Iruela-Arispe L, Begley U, Tang D, Galper JB: 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors interfere with angiogenesis by inhibiting the geranylgeranylation of RhoA. *Circ Res* 2002; **91**: 143–150.
37. Cai W, Chen X: Anti-angiogenic cancer therapy based on integrin alphavbeta3 antagonism. *Anticancer Agents Med Chem* 2006; **6**: 407–428.
38. Ruegg C, Mariotti A: Vascular integrins: pleiotropic adhesion and signaling molecules in vascular homeostasis and angiogenesis. *Cell Mol Life Sci* 2003; **60**: 1135–1157.
39. Higuchi M, Yasuda O, Kawamoto H, *et al*: Tissue inhibitor of metalloproteinase-3 deficiency inhibits blood pressure elevation and myocardial microvascular remodeling induced by chronic administration of N^{ω}-nitro-L-arginine methyl ester in mice. *Hypertens Res* 2007; **30**: 563–571.
40. Tan J, Hua Q, Xing X, Wen J, Liu R, Yang Z: Impact of the metalloproteinase-9/tissue inhibitor of metalloproteinase-1 system on large arterial stiffness in patients with essential hypertension. *Hypertens Res* 2007; **30**: 959–963.