Inhibition of Migration and Proliferation of Rat Vascular Smooth Muscle Cells by a New HMG-CoA Reductase Inhibitor, Pitavastatin

Masakazu KOHNO, Kaori SHINOMIYA, Satomi ABE, Takahisa NOMA, Isao KONDO, Akira OSHITA, Hiroto TAKEUCHI, Yuichiro TAKAGI, Kazushi YUKIIRI, Katsufumi MIZUSHIGE, and Koji OHMORI

The migration and proliferation of vascular smooth muscle cells (SMCs) are known to play roles in the pathogenesis of atherosclerosis. Therapy with a reductase inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) (‘statin’) produces significant alterations in various SMC functions. The objectives of the present study were to determine whether pitavastatin, a new chemically synthesized and powerful statin, can affect angiotensin II (Ang II)- and platelet-derived growth factor (PDGF)-induced migration and proliferation of cultured rat vascular SMCs. The effect of pitavastatin on cell viability was also examined in these cells. Migration was evaluated by the Boyden’s chamber method using microchemotaxis chambers. As expected, Ang II and PDGF BB potently stimulated cell migration in a concentration-dependent manner. Pitavastatin significantly inhibited Ang II (10^{-6} mol/l)-induced migration at the concentrations of 10^{-8} and 10^{-7} mol/l. Pitavastatin also inhibited PDGF BB (1 ng/ml)-induced migration at concentrations between 10^{-9} and 10^{-8} mol/l in a relatively concentration-dependent manner. This statin modestly but significantly inhibited Ang II (10^{-6} mol/l)- and PDGF BB (1 ng/ml)-induced DNA synthesis at concentrations between 10^{-9} and 10^{-7} mol/l. In addition, pitavastatin clearly inhibited Ang II (10^{-6} mol/l)- and PDGF BB (1 ng/ml)-induced increases of cell number at concentrations between 10^{-8} and 10^{-7} mol/l. Pitavastatin did not affect lactate dehydrogenase release from these cells at the concentrations used in this experiment. In a trypan blue exclusion test, dead cells stained with trypan blue were not found 24 h after treatment with 10^{-9}, 10^{-8} or 10^{-7} mol/l of pitavastatin. These findings suggest that pitavastatin suppresses the migration and proliferation stimulated by Ang II and PDGF BB without affecting cell viability. Pitavastatin may exert an anti-atherogenic effect, in part, through these mechanisms. (Hypertens Res 2002; 25: 279–285)

Key Words: migration, proliferation, pitavastatin, angiotensin II, PDGF

Introduction

Pitavastatin has recently been developed as a new chemically synthesized and powerful inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) (‘statin’) (1–3).

Recent clinical and experimental studies suggest that statins exert some direct anti-atherogenic effects as well as cholesterol-lowering effects (4–7). In fact, there exists in vivo and in vitro evidence of decreased smooth muscle cell (SMC) proliferation after administration of fluvastatin, simvastatin or lovastatin (8). Recently, the migration stimulated by
lyso phosphatidylcholine, a major phospholipid component of oxidized low density protein (LDL), was shown to be suppressed by fluvastatin, simvastatin or pravastatin in cultured human coronary artery SMCs (9). Further, pitavastatin has been shown to strongly suppress neointimal thickening by inhibiting SMCs proliferation and fibronectin production in a balloon-injured rabbit carotid artery model (10).

On the other hand, the migration of medial SMCs into the intima and the proliferation of migrated cells are proposed to be key processes of intimal thickening in atherosclerotic lesions (11–13). Previously, we have shown that angiotensin II (Ang II) and platelet-derived growth factor (PDGF) stimulate migration and proliferation of SMCs via Ang II type 1 (AT1) receptors and PDGF β receptors, respectively (14–16). In particular, the importance of the renin-angiotensin system in SMCs proliferation and migration has been established in intimal lesion formations after balloon injury (17–23). Both angiotensin-convertase enzyme inhibitors and AT1 receptor antagonists have been shown to reduce intimal thickening in balloon injury models (19, 20).

Accordingly, we here examined the effects of a new statin, pitavastatin, on Ang II- and PDGF BB-induced migration and proliferation of cultured rat vascular SMCs. In addition, we also examined the effect of pitavastatin at the concentrations used in the current experiment on cell viability as measured by lactate dehydrogenase (LDH) release from these cells and a trypan blue exclusion test.

**Methods**

**Materials**

Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum (FCS), trypsin, Versine, penicillin, and streptomycin were purchased from GIBCO Lab. (Gaithersburg, USA). Bovine serum albumin (BSA) and PDGF (recombinant BB) were purchased from Sigma Chemical Co. (St. Louis, USA). Flasks and multiwell plates were purchased from Becton Dickinson (Rockville, USA). Diff-Quick staining solution was purchased from Green-Cross Corp. (Kyoto, Japan). Type I collagen was purchased from Koken Inc. (Tokyo, Japan). Pitavastatin was the generous gift of Kowa Co., Ltd. (Tokyo, Japan).

**Culture of SMCs**

All studies were performed using protocols approved by the Animal Review Committee of Kagawa Medical University. Rat vascular SMCs were grown from the aortic explants of Sprague-Dawley rats and were cultured in DMEM containing 10% FCS, penicillin (50 U/ml), and streptomycin (50 µg/ml) as previously described (24). Cells were identified as SMCs according to their morphological and growth characteristics (24). Cultures were maintained at 37°C in a mixture of atmospheric air and 5% CO2. Cells were subcultured after treatment with 0.25% trypsin and 0.02% EDTA. Subconfluent SMCs between the 4th and 8th passages were used for the experiments.

**Evaluation of Cell Migration**

Migration of SMCs was assayed by a modification of Boyden’s chamber method using microchemotaxis chambers (Neuro Probe Inc., Oxnard, USA) and polycarbonate filters (Nucleopore Corp., Oxnard, USA) with pores of 5.0 µm in diameter, as previously reported (24). Cultured SMCs were trypsinized and suspended at a concentration of 5.0 × 10^6/ml in DMEM supplemented with 0.4% BSA. The cell number was counted with an electronic cell counter (Model ZB1; Coulter Electronics, Grand Island, USA). A volume of 200 µl of SMC suspension was placed in the upper chamber, and 40 µl of DMEM/0.4% BSA containing migration factors such as Ang II or PDGF BB were placed in the lower chamber. The chamber was incubated at 37°C under 5% CO2 in air for 8 h, as previously described. After incubation, SMCs on the upper side of the filter were scraped off and the filter was removed. The SMCs that had migrated to the lower side of the filter were fixed in ethanol, stained with Diff-Quick staining solution, and counted under a microscope (magnification, × 400) for quantitation of SMC migration. Migration activity was calculated as the mean number of migrated cells observed in four high-power fields and given as the mean value of four measurements.

**Evaluation of Cell Proliferation**

SMCs proliferation was evaluated according to the Biotrak™ cell proliferation ELISA system (Amersham Pharmacia Biotech, St. Louis, USA). This technique is based on the measurement of 5-bromo-2′-deoxyuridine (BrdU) incorporation instead of thymidine during DNA synthesis in proliferating cells. After 24 h of SMC seeding in a 96-well microplate at 37°C, the medium was exchanged with medium containing 10^-9, 10^-8 or 10^-7 mol/l pitavastatin. Then cells were reincubated for 48 h. BrdU was added and the cells were reincubated for 4 h. After removing the culture medium, SMCs were fixed and the DNA denatured. Then peroxidase-labelled anti-BrdU was added to bind to the BrdU. The immune complexes were detected by the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate reaction, and the resultant color was read at 450 nm in a microplate spectrophotometer. The absorbance values correlated directly to the amount of DNA synthesis and thereby to the number of proliferating cells in culture.

**Evaluation of Cell Viability**

LDH release was evaluated according to our previously described method (25). LDH was measured in cell culture supernatants after 24 h of incubation at 37°C in DMEM with or with-
out varying concentrations of pitavastatin (10^{-9}, 10^{-8} or 10^{-7} mol/l). LDH release was expressed as a percent change compared with the control.

In the trypan blue exclusion test, dead cells stained with trypan blue were evaluated 24 h after the addition of 10^{-9}, 10^{-8} or 10^{-7} mol/l pitavastatin to the medium.

Calculations and Analysis

The statistical significance of differences in the results was evaluated using an unpaired analysis of variance, and probability values were calculated by Scheffe’s method (26). All values were expressed as the mean ± SD.

Results

Effects of Pitavastatin on SMC Migration

The stimulatory effects of Ang II and PDGF BB on migration are shown in Table 1. As expected, Ang II and PDGF BB stimulated migration in a concentration-dependent manner.

Figure 1A shows the effects of various concentrations of pitavastatin on Ang II-stimulated migration. The migration-stimulatory effects of 10^{-6} mol/l Ang II were significantly inhibited by 10^{-8} and 10^{-7} mol/l pitavastatin. Figure 1B shows the effects of various concentrations of pitavastatin on PDGF BB-stimulated migration. The migration-stimulatory effects of 1 ng/ml PDGF BB were significantly inhibited by 10^{-9} and 10^{-7} mol/l pitavastatin in a relatively concentration-dependent manner.

Effects of Pitavastatin on SMC Proliferation

Figure 2 shows the effects of various concentrations of pitavastatin on the Ang II (10^{-6} mol/l)- and PDGF BB (1 ng/ml)-induced increases of DNA synthesis. Pitavastatin modestly but significantly inhibited the Ang II- and PDGF BB-induced increases of DNA synthesis at concentrations between 10^{-9} and 10^{-7} mol/l.

Effects of Pitavastatin on Cell Viability

The effects of pitavastatin on cell viability were assessed on the basis of LDH release from these cells. However, LDH release was not increased by either 10^{-9}, 10^{-8} or 10^{-7} mol/l pitavastatin.
pitavastatin compared with that in controls (Fig. 4).

Furthermore, in a trypan blue exclusion test, dead cells stained with trypan blue were not found 24 h after treatment with $10^{-9}$, $10^{-8}$ or $10^{-7}$ mol/l pitavastatin (Fig. 5).

**Discussion**

In the present study, we have confirmed our previous findings that Ang II and PDGF BB induced rat or human vascular SMC migration (14–16, 21). We have also shown that a new HMG-Co A reductase inhibitor, pitavastatin, clearly suppressed Ang II- and PDGF BB-induced SMC migration at reported therapeutic concentrations ($10^{-9}$ mol/l) (27). Pitavastatin modestly reduced Ang II- and PDGF BB-induced DNA synthesis and clearly suppressed the Ang II- and PDGF BB-induced increase of cell number at the therapeutic concentrations. Kitahara et al. have previously demonstrated that pitavastatin suppresses neointimal thickening induced by balloon injury at the doses used in lipid lowering (10). The SMC migration from media to intima and the growth in the
intimal lesion occur in response to vascular injury \((11-13)\). Endothelial denudation by a balloon catheter causes neointimal thickening with SMC migration and growth in the intima \((28, 29)\). Taken together with our findings, these observations suggest that pitavastatin may suppress restenosis after angioplasty through inhibition of both SMC migration and proliferation. Although we have not analyzed other statins in this experiment, the effect of pitavastatin on the intimal thickening after balloon injury is shown to more potent than that of simvastatin or pravastatin \((10)\).

The cellular mechanism of pitavastatin in SMC migration is not clear at present. Among the possibilities, the effect of pitavastatin on protein kinase C (PKC) activity is interesting. We have previously shown that the activation of PKC is, at least in part, related to cell migration \((25)\). Actually, PKC inhibitors reduced the cell migration stimulated by various vasoactive substances \((25)\). In addition, in PKC-depleted cells, cell migration was markedly reduced \((25)\). Recently Yasunari et al. have demonstrated that the HMG-CoA reductase-inhibitors directly suppress phospholipase D activity, leading to the suppression of membrane-binding PKC activity \((9)\). This effect of HMG-CoA reductase inhibitors appears to be related to their inhibition of prenylation of heterotrimeric and low-molecular-weight guanosine triphosphate-binding proteins that are involved in signal transduction \((30)\). A second possibility is that pitavastatin inhibits SMC migration and proliferation through inhibition of mevalonate production. In this context, Habenichi et al. and Soma et al. have previously shown that simvastatin inhibits SMC migration and growth through inhibition of mevalonate production under \textit{in vivo} and \textit{in vitro} conditions \((31, 32)\). However, the effects of simvastatin on cell number, mitochondrial dehydrogenase activity and DNA synthesis are found to be counteracted by simultaneous mevalonate addition \((33)\). Clearly, further evaluation of these hypotheses will be needed.

In the present study, dead cells stained with trypan blue were not found \(24\) h after treatment with \(10^{-9}, 10^{-8}\) or \(10^{-7}\) mol/l pitavastatin. Furthermore, these concentrations of pitavastatin did not affect LDH release from the cells. Therefore, it is likely that the observed migration- and proliferation-inhibitory effects were not a result of cytotoxicity or diminished cell viability.

In conclusion, our present findings suggest that a new HMG-CoA reductase inhibitor, pitavastatin, inhibits Ang II- and PDGF BB-induced migration and proliferation without affecting cell viability. Pitavastatin may thus affect the early events of atherogenesis by suppressing increased SMC mi-
migration and proliferation induced by activation of the renin-angiotensin system and the coagulation cascade. Recently, pitavastatin was found to suppress the foam cell formation induced by modified LDL (34), and to inhibit the production of fibronectin and type I collagen (10). In addition, the cholesterol-lowering effect of pitavastatin has been shown to be equivalent to that of atrovastatin, which is the most potent of the currently available HMG-CoA reductase inhibitors (34). Therefore, pitavastatin may provide a new and valuable therapeutic agent for hypercholesterolemic patients. However, because this study was performed on cultured SMCs, caution must be exercised in extrapolating its findings to in vivo conditions. In addition, further studies will be needed to clarify the exact cellular mechanisms responsible for the actions and interactions of HMG-CoA reductase inhibitors.

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References


24. Kohno M, Yasunari K, Maeda K, et al: Effects of cardiac natriuretic peptides on oxidized low-density lipoprotein-


