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RESEARCH PAPER

Atherosclerosis induced by chronic inhibition of the synthesis of nitric oxide in moderately hypercholesterolaemic rabbits is suppressed by pitavastatin

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Background and purpose: It is not clear if the new 3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor pitavastatin prevents atherogenesis by a direct effect. Statins have a cholesterol-lowering effect, so an accessible animal model of atherosclerosis showing only moderate hypercholesterolaemia as in humans, is needed. The effects of pitavastatin were evaluated on atherosclerotic lesions accumulating foam cells derived from macrophages, produced in rabbits with moderate hypercholesterolaemia by chronic inhibition of nitric oxide synthase (NOS).

Experimental approach: White New Zealand rabbits were fed a 0.2% cholesterol diet with the NOS inhibitor N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME) in the same diet. Pitavastatin (0.1 and 0.3 mg·kg^{-1}) was given orally once a day for 8 weeks. The aortic arch and thoracic aorta were analysed by histochemistry and atherosclerotic lesions were quantified. The effect of pitavastatin on adhesion of THP-1 cells to endothelial cells, and cholesterol content in RAW264.7 cells incubated with oxidized or acetylated LDL were also investigated.

Key results: Atherosclerotic lesions containing foam cells were induced in a model of atherosclerosis in rabbits with moderate hypercholesterolaemia by chronic inhibition of NOS. The area of atherosclerotic lesions was diminished by pitavastatin administration. The adhesion of THP-1 cells and cholesteryl ester content in RAW macrophages were decreased by pitavastatin treatment.

Conclusion: Atherosclerosis induced by chronic inhibition of NOS in moderately hypercholesterolaemic rabbits was suppressed by pitavastatin via inhibition of macrophage accumulation and macrophage foam cell formation.
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Keywords: adhesion; atherosclerosis; lipid accumulation; L-NAME; macrophages; moderate hypercholesterolaemia; pitavastatin

Abbreviations: CMC, carboxymethylcellulose; HDL, high-density lipoprotein; HMG-CoA, developed 3-hydroxyl-3methylglutaryl coenzyme A; L-NAME, N$^{\omega}$-nitro-L-arginine methyl ester; LDL, low-density lipoprotein; NOS, nitric oxide synthase; Statin, (HMG-CoA) reductase inhibitor; TC, total cholesterol; TG, triglycerides; VLDL, very-low-density lipoprotein; WHHL, Watanabe heritable hyperlipidemic

Introduction

Large-scale primary and secondary prevention studies revealed that cholesterol-lowering therapy with inhibitors of 3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase (the statins) is effective against the development of acute coronary syndromes (The Scandinavian Simvastatin Survival Study Group, 1994; Shepherd *et al.*, 1995; Gotto, 1997). Atherosclerotic plaques that are prone to rupture have a thin fibrous cap covering macrophages and a lipid-rich core and such plaques can be stabilized by reducing the total cholesterol (TC) in serum (Aikawa *et al.*, 1998). Suppression of the atherosclerotic area is assumed to be the secondary effect of statins.

The new HMG-CoA reductase inhibitor pitavastatin (Aoki *et al.*, 1997) is more potent in lowering plasma cholesterol,

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compared with other statins (Suzuki *et al.*, 1999; Suzuki *et al.*, 2001). In clinical trials investigating patients with hyperlipidemia, pitavastatin was confirmed as lowering serum cholesterol and was considered safe (Kajinami *et al.*, 2000; Saito *et al.*, 2002; Yokote *et al.*, 2008).

Although atherosclerotic models in pigs are reported to be similar to human atherosclerosis because serum cholesterol level, blood pressure and heart rate of pigs are similar to those of humans (Bonetti *et al.*, 2002; Crespo *et al.*, 2005), pigs are not common experimental animals in many laboratories as they are too big to handle easily. Rabbits fed 0.3–1% cholesterol diet provide more common atherosclerotic models because they readily develop atherosclerotic lesions. However, in such animals, the serum cholesterol level is 10–20 $mg \cdot mL^{-1}$, much higher than that found in human hypercholesterolaemia. So, there is a need for an atherosclerotic model in rabbits, with only moderately raised serum cholesterol to levels similar to those found in humans.

Administration of pitavastatin in drinking water ($0.5\ mg \cdot kg^{-1}$) suppressed the progression of atherosclerosis by reducing TC and triglyceride (TG)-rich lipoprotein in Watanabe heritable hyperlipidemic (WHHL) rabbits (Suzuki *et al.*, 2000). One of the reasons for the stabilization of atherosclerotic plaques was that pitavastatin reduced the areas reacting positive for monocyte chemoattractant protein, matrix metalloproteinase (MMP)-3 and MMP-9 by 39%, 41% and 52% respectively (Suzuki *et al.*, 2003). WHHL rabbits are models for familial hypercholesterolaemia and defects in the function of the low-density lipoprotein (LDL) receptor, so the level of TC in the serum of WHHL rabbits is higher than that in general hypercholesterolaemia in humans. Serum cholesterol levels were also decreased by pitavastatin administration. Pitavastatin markedly retarded the progression of atherosclerosis during hypercholesterolaemia by improving the bioactivity of nitric oxide (NO) in oophorectomized rabbits, given a 0.3% cholesterol diet (Hayashi *et al.*, 2004). This model also has a much higher level of TC in serum and reduced level of cholesterol due to pitavastatin under hormonal regulation. Whether the reduction in atherosclerotic area is a direct effect of pitavastatin is unknown. To clarify the direct effect of pitavastatin in the initial step of atherosclerosis progression (i.e. macrophage accumulation), it is necessary to create an animal model that is close to human atherosclerosis and which is accompanied by moderate hypercholesterolaemia.

Chronic inhibition of nitric oxide synthase (NOS) by N^{ω}-nitro-L-arginine methyl ester (L-NAME, $12\ mg \cdot day^{-1}$) using an osmotic pump promoted early neointima formation in the thoracic aorta of rabbits fed a 0.5% cholesterol diet,because monocyte recruitment or accumulation of foam cell lipids were modified (Cayatte *et al.*, 1994). In this animal model, serum TC levels were also very high compared with human moderate hypercholesterolemia although the TC in the serum of L-NAME-treated rabbits was the same as that in non-treated rabbits. Oral administration of L-NAME in drinking water to rabbits fed a 0.5% cholesterol diet for 8 months failed to promote atherosclerotic lesions in the aorta and the coronary artery (Nakamura *et al.*, 1998). This was despite demonstrable inhibition of NOS in the arterial endothelium and a decreased NO_3 concentration in serum (Nakamura *et al.*, 1998). To create an animal model which allows macrophage accumulation in the aorta without an increase in the level of serum cholesterol, it is essential to regulate the quantity of dietary cholesterol and the dose of L-NAME.

We have developed a rabbit atherosclerosis model in which macrophages are accumulated in the vessel wall under moderate hypercholesterolaemia, with chronic inhibition of NOS by L-NAME in the diet. Using this model, we investigated if pitavastatin directly suppressed accumulation of cholesterol and lipid-laden macrophages.

Methods

Animals, feeding and planning of administration of L-NAME and pitavastatin

Animal care and experimental procedures were carried out in accordance with the Guidelines of the Animal Investigation Committee, Materials and Chemical Research Laboratories of Nissan Chemical Industries Limited (Saitama, Japan).

Male white New Zealand rabbits (2.5–2.7 kg) were obtained from Kitayama Laboratories (Ina, Nagano, Japan) and cholesterol supplemented diet was obtained Oriental Yeast Co., Ltd. (Tokyo, Japan). A 0.2% cholesterol-supplemented diet containing L-NAME (Sigma-Aldrich, St. Louis, MO, USA) was prepared in our laboratory, as follows. Briefly, L-NAME was initially dissolved in a minimum volume of water and diluted to a 70% ethanol solution. This solution was scattered on 0.2% cholesterol chow by a sprayer so that the final content of L-NAME was 0.075%, 0,125% and 0.175% (v/v). The chow was dried under shading overnight at 25°C and given to rabbits the next day.

To create an animal model of atherosclerotic plaques accompanied by moderate hypercholesterolaemia and without raising serum cholesterol level by L-NAME, the cholesterol feeding and L-NAME administration was carried out as shown in Figure 1. Twenty-four rabbits were given this diet ($100\ g \cdot day^{-1}$) throughout the experimental period. Rabbits were initially fed 1% cholesterol chow during the first week to immediately increase serum cholesterol level. Then the L-NAME dosage was incrementally increased as follows. Rabbits were changed to the 0.2% cholesterol diet containing 0.075% L-NAME (approximately $30\ mg \cdot kg^{-1} \cdot day^{-1}$) for the first 3 days, and switched to the 0.2% cholesterol diet containing 0.125% L-NAME (approximately $50\ mg \cdot kg^{-1} \cdot day^{-1}$) for the second 3 days. On the seventh day from the beginning of L-NAME administration, rabbits were given a 0.2% cholesterol diet containing 0.175% L-NAME (approximately $70\ mg \cdot kg^{-1} \cdot day^{-1}$) for 8 weeks.

At this time (the seventh day; see Figure 1), the animals were divided into three groups to investigate the effect of pitavastatin on atherosclerosis. Pitavastatin calcium salt (Nissan Chemical Industries Limited, Saitama, Japan) dissolved in 0.5% carboxymethylcellulose (CMC) was given orally once a day before feeding, from the seventh day to the end of the experimental period at doses of $0.1\ mg \cdot kg^{-1}$ and $0.3\ mg \cdot kg^{-1}$. Controls were given 0.5% CMC without pitavastatin.

Blood samples were obtained from ear vein at the times indicated in Figure 1. Serum TC and TG were measured by

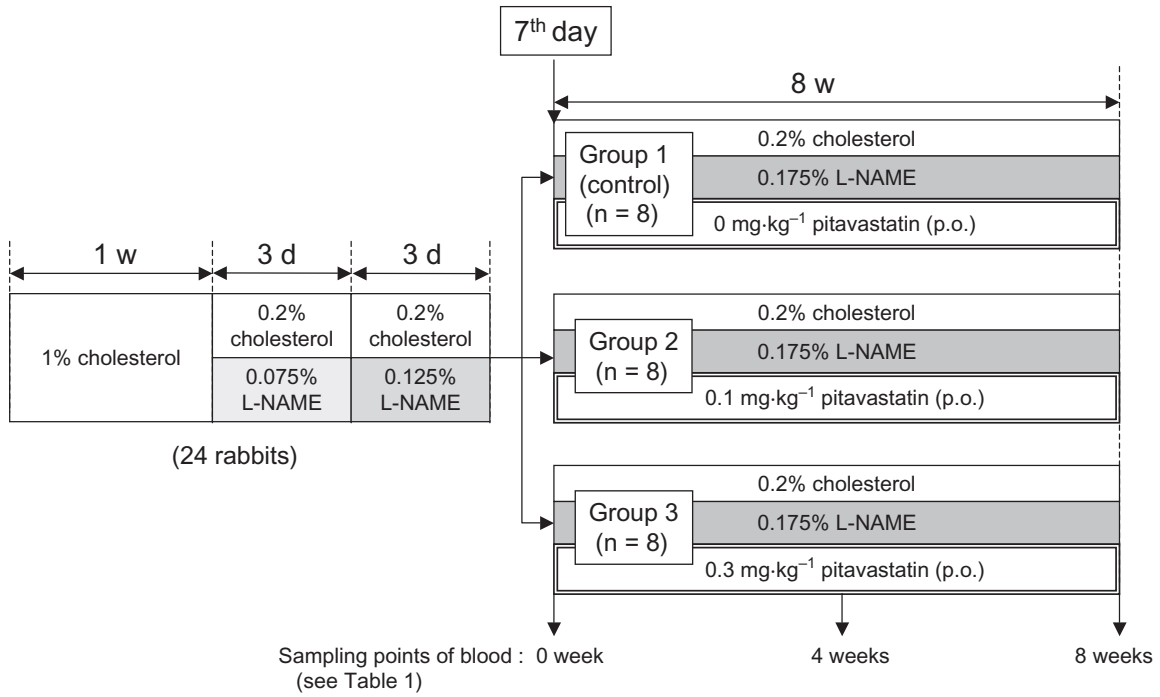

Figure 1 Experimental schedule of cholesterol feeding and administration of L-NAME and pitavastatin. The feeding regimen of cholesterol chow and administration of L-NAME and pitavastatin is summarized. Rabbit were fed a cholesterol diet containing L-NAME and pitavastatin was given orally once a day. L-NAME, N^{ω}-nitro-L-arginine methyl ester.

Determiner TC and Determiner TG (Kyowa Medica; Tokyo, Japan). High-density lipoprotein (HDL) cholesterol was measured by precipitation methods using phosphotungstic acid magnesium (Wako Pure Chemical Industries, Limited, Osaka, Japan) followed by TC measurement as described below. At the end of the experimental period, rabbits were anaesthetized with pentobarbital (50 mg·kg^{-1} Nembutal, Dainippon Sumitomo Pharma, Osaka, Japan) and killed by exsanguination. Proximal and distal portions of the aortic arch were excised and examined by histochemical analysis.

Chemical staining and immunohistochemistry

The aortic arch and thoracic aorta were stained with Sudan III and the lesion area measured. Cross-sections of the aortic arch and thoracic aorta were stained with haematoxylin–eosin (H&E). To determine the lipid deposits in the lesions, frozen cross-sections of the aortic arch were stained with Oil Red O. Paraffin-embedded cross-sections of formalin-fixed tissues were stained with anti-macrophage antibody (RAM11) and anti-smooth muscle cell antibody (HHF35), and counterstained with Mayer's haematoxylin solution (Wako). The lesion area was measured using a computer-based image analyser system (Luzex 3U: Nireco; Tokyo, Japan) without knowledge of the treatments received.

Lipoproteins

Human LDL was prepared from fresh serum by ultracentrifugation at $1.006 < d < 1.063$ and maintained under a nitrogen atmosphere. LDL acetylation was achieved by addition of acetic anhydride according to the method of Basu *et al.* (1976). LDL was oxidized by 2 h exposure to 254 nm UV light according to the method of Dousset *et al.* (1990).

Adhesion of monocytes to endothelial cells

Human umbilical vein endothelial cells (HUVECs) were maintained on gelatin-coated black 48-well plates in endothelial cell basal medium (EBM; Bio Whittaker Incorporated, Walkersville, MD, USA) containing 5% fetal bovine serum (FBS; Bio Whittaker) and supplements [human epidermal growth factor (EGF), hydrocortisone, bovine brain extract, and gentamicin]. They were stimulated with 10 ng·mL^{-1} tumour necrosis factor-α (TNF-α) for 8 h. Human acute monocytic leukemia (THP-1) cells (American Type of Cell Culture, Rockville, MD, USA) or HUVECs were maintained in RPMI-1640 medium (Iwaki, Asahi Glass Company Limited, Funabashi, Japan) containing 10% FBS, and were treated with pitavastatin for 48 h before adhesion assays. Thirty minutes before assay, THP-1 cells were pre-labelled with BCECF-AM (Wako). BCECF-AM-labelled THP-1 cells were suspended in fresh RPMI-1640 medium at 10^6 cells·mL^{-1}, and 0.2 mL of the cell suspension (2×10^5 cells) was loaded on TNF-α-treated HUVECs. After 2 h of culture, non-adherent THP-1 cells were removed by washing three times with RPMI-1640 medium. The fluorescence of adherent THP-1 cells was measured with a fluorescence microplate reader (Excitation, 485 ± 20 nm; Emission, 530 ± 25 nm). HUVECs were treated with Y-27632 (1–10 μM) or 0.15 μg·mL^{-1} Toxin B for 1 h and treated with TNF-α for 8 h. THP-1 cells were treated with Y-27632 (0.3–1 μM) or 0.15 μg·mL^{-1} Toxin B for 8 h before adhesion assay, and loaded on HUVECs. The expression of ICAM-1 or VCAM-1 on HUVECs after 8 h treatment with TNF-α was assessed by

cell-ELISA, using anti-ICAM-1 antibody (BBIG-I1, R&D System inc, Minneapolis, MN, USA) or anti-VCAM-1 antibody (BBIG-V1, R&D System inc).

Formation of macrophage foam cells

RAW264.7 mouse peritoneal macrophages (American Type of Cell Culture) were maintained in 12-well culture plates in Dulbecco's modified Eagle's medium (DMEM, Iwaki, Japan) containing 10% FBS. Cells were cultured with pitavastatin, simvastatin or atorvastatin for 48 h. Cells were exposed to oxidized LDL or acetylated LDL containing 200 μg cholesterol·mL^{-1} and the abovementioned HMG-CoA reductase inhibitors for 2 days. Cell numbers were determined by a Coulter counter (Coulter; ZM1) and cell lipids extracted by hexane : 2-propanol (2:1). Lipid extracts were transferred to 96-well plates with 1% Triton X-100/EtOH. TC and free cholesterol (FC) were measured by Determiner TC and Determiner FC (Kyowa Medica).

Statistical analysis

Data are shown as means ± SE for animal studies and means ± SD for cell culture studies. Statistical significance was evaluated by Dunnett's multiple parameter analysis or Student's *t*-test using SAS or super ANOVA.

Materials

The TNF was obtained from Roche Diagnostics, Basel, Swizerland; Y27632 from BIAFFIN GmbH & Co KG, Kassel, Germany and Toxin B from Calbiochem-Merck4Biosciences, Merck KGaA, Darmstadt, Germany. Mevalonate lactone, geranylgeraniol and farnesol were from Sigma.

Results

Effect of pitavastatin on levels of lipid in serum

The rabbits consumed all the food every day, and there were no difference in body weight among the three groups, either at the start (control, 2.77 ± 0.05 kg; 0.1 mg·kg^{-1} pitavastatin, 2.76 ± 0.05 kg; and 0.3 mg·kg^{-1} pitavastatin, 2.80 ± 0.05 kg) or at the end of the experiment (control, 2.97 ± 0.08 kg: 0.1 mg·kg^{-1} pitavastatin, 2.88 ± 0.06 and 0.3 mg·kg^{-1} pitavastatin, 2.89 ± 0.08 kg).

The effects of pitavastatin on the levels of TC, HDL-C and TG in serum are shown in Table 1. Serum levels of TC and TG were similar among all groups before starting pitavastatin administration (0 week in Table 1). After feeding of a 1% cholesterol diet for 1 week, serum TC level in all rabbits was approximately 7 mg cholesterol·mL^{-1} After change to a 0.2% cholesterol diet, the serum level of TC decreased slowly and was stable after 4 weeks. A diet containing 0.2% cholesterol achieved moderate hypercholesterolaemia (cholesterol concentration of ~3.5 mg·mL^{-1}) and the presence of L-NAME did not change the cholesterol level in serum. Serum HDL-C slightly (but not significantly) increased in rabbits treated with pitavastatin (0.3 mg·kg^{-1}).

Table 1 Effect of pitavastatin on serum cholesterol, HDL-cholesterol and triglyceride levels

Groups	*Total cholesterol (mg·mL^{-1})*			*HDL cholesterol (mg·mL^{-1})*			*Triglycerides (mg·mL^{-1})*			
	0 week	*4 weeks*	*8 weeks*	*0 week*	*4 weeks*	*8 weeks*	*0 week*	*4 weeks*	*8 weeks*	
Contorol	4.34 ± 0.49	3.73 ± 0.95	3.53 ± 1.03	0.25 ± 0.06	0.25 ± 0.09	0.24 ± 0.08	0.13 ± 0.02	0.48 ± 0.14	0.46 ± 0.10	*
Pitavastatin (0.1 mg·kg^{-1})	4.65 ± 0.66	2.97 ± 0.81	3.66 ± 0.62	0.26 ± 0.05	0.24 ± 0.08	0.24 ± 0.08	0.13 ± 0.04	0.25 ± 0.60	0.36 ± 0.10	
Pitavastatin (0.3 mg·kg^{-1})	4.47 ± 0.60	3.33 ± 0.58	3.74 ± 0.56	0.25 ± 0.04	0.25 ± 0.06	0.28 ± 0.06	0.12 ± 0.02	0.21 ± 0.06	0.21 ± 0.03	

*$P < 0.05$, significantly different from control (Dunnett's test).
Rabbits were treated as described in Figure 1. Blood samples were collected before (0 week) and after the fourth week of pitavastatin administration, and the end of experimental period (8 weeks), as shown in Figure 1.
Each value represents mean ± SE.
HDL, high-density lipoprotein.

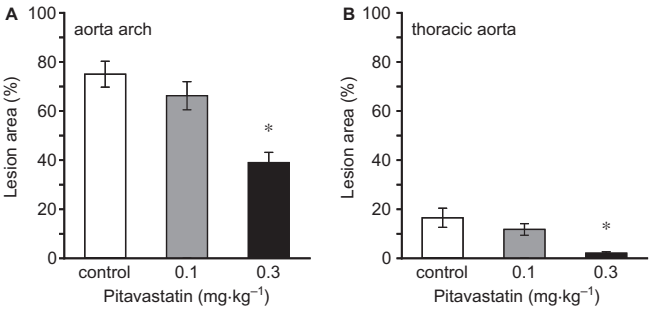

Figure 2 Effect of pitavastatin on the area of atherosclerotic surface lesions in the aortic arch (A) and thoracic aorta (B). Rabbits were treated with 0.1 and 0.3 mg·kg^{-1} pitavastatin once a day during the experimental period. The aortic arch and thoracic aorta were stained with Sudan III. Each column represents mean ± SE. Asterisk (*) indicates significant difference from control at $P < 0.05$ by Dunnett's test.

In the control group, serum TG level increased in the fourth and eighth week of the experimental period, but serum TC did not change. At the eighth week, serum TG level was significantly lower in rabbits treated with pitavastatin (0.3 mg·kg^{-1}), but serum TC did not change.

Effect of pitavastatin on atherogenesis

When rabbits were fed a diet containing L-NAME, the area of atherosclerotic surface lesion was 0.33 ± 0.13% and the intima/media ratio was 0.09 ± 0.03 in aortic arch. In rabbits fed a diet containing 0.3% cholesterol, the corresponding area was 55.4 ± 19.6% and the intima/media ratio was 0.20 ± 0.07 in the aortic arch (data not shown), but serum cholesterol level was over 8 mg·mL^{-1} which was much higher than in human hypercholesterolaemia. Rabbits fed 0.2% cholesterol diet containing L-NAME developed moderate hypercholesterolaemia and showed increased atherosclerotic lesions, including surface involvements at 75.0 ± 5.3% in the aortic arch and 16.5 ± 3.9% in the thoracic aorta (Figure 2), as well as intima/media ratio at 0.30 ± 0.05 in the aortic arch and 0.32 ± 0.12 in the thoracic aorta (Figure 4).

Percentages of the area of atherosclerotic surface lesions in the aorta and thoracic aorta of rabbits treated with pitavastatin are shown in Figure 2. The area in the aortic arch and thoracic aorta decreased in a dose-dependent manner after treatment with pitavastatin, and the lesion areas of rabbits treated with 0.3 mg·kg^{-1} pitavastatin were significantly lower than those of controls. Figure 3A,B are H&E-stained cross-sections of the aortic arch in the control group and the 0.3 mg·kg^{-1} pitavastatin group respectively. Pitavastatin suppressed thickening of the neointimal lesion area, and the intima/media ratio in the aortic arch and thoracic aorta decreased after treatment with pitavastatin at the higher dose (0.3 mg·kg^{-1}; Figure 4A,D).

Photographs of histochemical staining of the cross-section of the aortic arch by Oil Red O staining, RAM11 staining and HHF35 are shown in Figure 3. In the control group, lipid deposits (Oil Red O) and increased numbers of macrophages (RAM11) were observed in the intimal lesion, particularly enhanced in the luminal fibrous cap portion. There was a

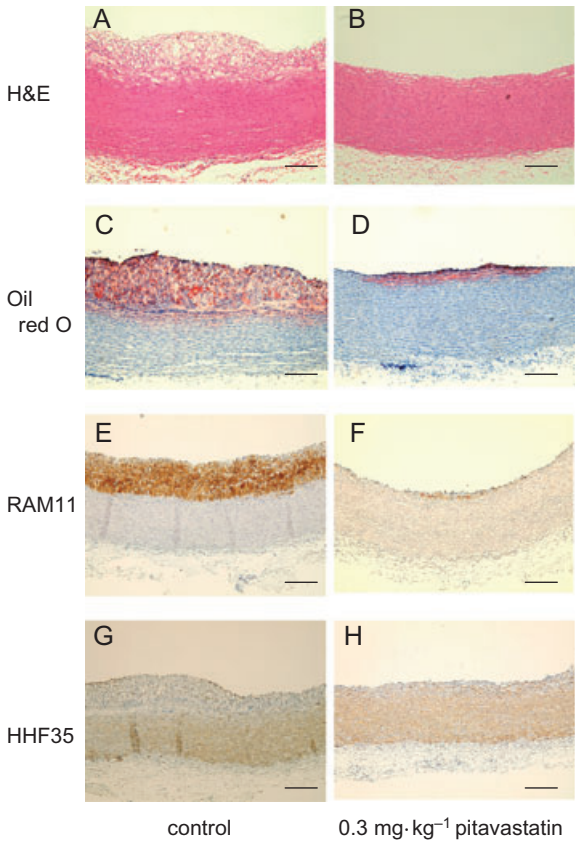

Figure 3 Cross-sections from a typical aortic arch. Rabbits were treated as described in Figure 1. Panels A and B, H&E staining; panels C and D, Oil Red O staining; panels E and F, immunostaining using anti-macrophage antibody (RAM11); panels G and H, immunostaining using anti-smooth muscle actin antibody (HHF35). Panels A, C, E and G were control rabbits and panels B, D, F and H were pitavastatin (0.3 mg·kg^{-1})-treated rabbits. Bar denotes 200 μm in each photograph. H&E, haematoxylin–eosin.

high degree of co-localization of lipid deposition and macrophages in the intimal lesion. Smooth muscle cells (immunostained with HHF35) were mainly seen in the medial lesion and a small part of the deep portion of the intima. Pitavastatin treatment diminished the area of lipid deposits (stained with Oil Red O; Figure 4B,E), with significant effects in thoracic aorta after treatment with pitavastatin (0.3 mg·kg^{-1}). Macrophage-positive areas (immunostained with RAM 11) were reduced by pitavastatin (0.3 mg·kg^{-1}) but these effects were not significant in either thoracic aorta or aortic arch samples (Figure 4C,F). Few areas were stained with HHF35 in the intima, and these were not detected by the image analyser, so there are no data for the HHF35-stained area to be shown in Figure 4. Overall, our results suggested that atherosclerotic lesions in this model may be primarily dependent on macrophage accumulation but not accumulation of smooth muscle cells, and that pitavastatin may prevent accumulation of macrophages and cholesterol in the neointima.

Effect of pitavastatin on adhesion of THP-1 cells to HUVECs

To clarify if pitavastatin influenced the adhesion of macrophages to the neointima, the number of THP-1 cells treated with

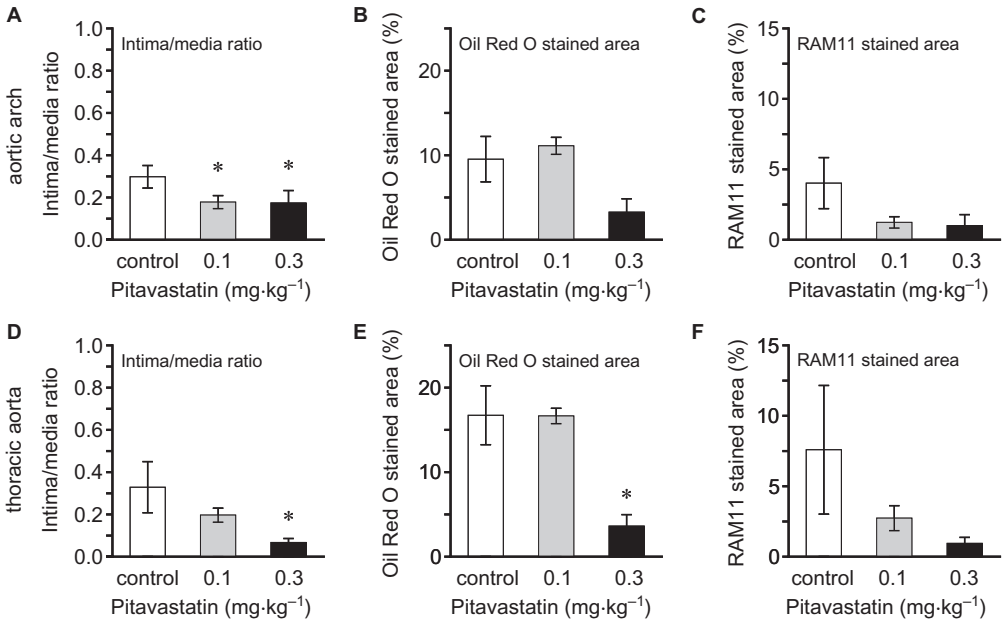

Figure 4 Quantification of the atherosclerotic areas of cross-sections in the aortic arch and thoracic aorta. The ratio of intima/media was calculated by measurement of each area of H&E-stained cross-section using LUZEX. Quantitative analysis of areas of lipid deposition and macrophage content was performed in Oil Red O-stained cross-sections and RAM11-immunostained cross-sections by LUZEX respectively. Graphs A–C show the data from the aortic arch samples, and graphs D–F that from the thoracic aorta samples. A and D, ratio of intima/media; B and E, percentage of Oil Red O-stained area; C and F, percentage of RAM 11-stained area. Each column represents mean ± SE. Asterisk (*) indicates significant difference from control at $P < 0.05$ by Dunnett's test.

pitavastatin adhering to HUVECs was measured. The adherence of THP-1 cells to HUVECs was significantly decreased by pitavastatin in a concentration-dependent manner (Figure 5A). When HUVECs were treated with pitavastatin, the inhibitory effect of pitavastatin was observed only at the highest concentration (1000 nM), and when THP-1 cells were treated with pitavastatin, adhesion of THP-1 cells was inhibited at lower concentrations of pitavastatin (Figure 5A). Cell surface expression of ICAM-1 in HUVECs was increased by 0.2 μM pitavastatin and decreased by 2 μM pitavastatin, and that of VCAM-1 was decreased by 0.2–2 μM pitavastatin (Figure 5B). When THP-1 cells were treated with pitavastatin in combination with mevalonic acid lactone or geranylgeraniol, the number of adherent cells remained at control levels (Figure 5C). The inhibitory effect of pitavastatin was not changed by addition of farnesol (Figure 5C). Atorvastatin also showed an inhibitory effect at 600 nM on THP-1 cell adhesion to HUVECs, but the effect was not significant (Figure 5C). The inhibitor of Rho-dependent kinase, Y-27632, and Toxin B also decreased THP-1 cell adhesion to HUVECs (Figure 5D,E).

Formation of macrophage foam cells

Pitavastatin suppressed the intra-cellular accumulation of cholesteryl ester, induced by oxidized LDL, in a concentration-dependent manner with an IC_{50} value of 56.3 nM. This inhibition was greater for the accumulation induced by oxidized LDL than for that induced by acetylated LDL (IC_{50}, 116 nM; Figure 6B). Pitavastatin was more potent than atorvastatin in these assays (IC_{50} for atorvastatin with oxidized LDL = 2200 nM; IC_{50} with acetylated LDL = 2409 nM).

The effects of pitavastatin on the formation of foam cells, derived from RAW macrophages, induced by oxidized LDL or acetylated LDL, were completely attenuated by addition of mevalonic acid lactone (see Table 2). After stimulation by oxidized LDL, the effects of pitavastatin were markedly attenuated by farnesol but less attenuated by geranylgeraniol (Table 2). After stimulation by acetylated LDL, the inhibitory effect of pitavastatin was partially and equally attenuated by geranylgeraniol and farnesol (Table 2).

Discussion

We have developed a short-term atherosclerosis model by chronic treatment with L-NAME in the diet of rabbits with moderate hypercholesterolaemia. In this model, foam cells mainly derived from macrophages are present in the atherosclerotic lesions.

Serum TG was elevated by chronic administration of L-NAME (Table 1), but was suppressed by administration of pitavastatin even though the serum cholesterol level did not change. Serum TG level was increased by L-NAME because of suppression of hepatic carnitine-palmitoyl CoA transferase (CPT) activity by chronic inhibition of NOS, which leads to elevated serum TG in rats (Khedara *et al.*, 1996; 1999). Conversely, we have reported that pitavastatin lowered TG levels in several experimental models (Suzuki *et al.*, 1999; 2000; 2001; Aoki *et al.*, 2002), and that pitavastatin suppressed secretion of hepatic very-low-density lipoprotein (VLDL) and the activity of intestinal microsome triglyceride transfer protein (MTP) (Ooyen *et al.*, 1999; Yamamoto *et al.*, 1999; Suzuki *et al.*, 2001). These data indicate that chronic

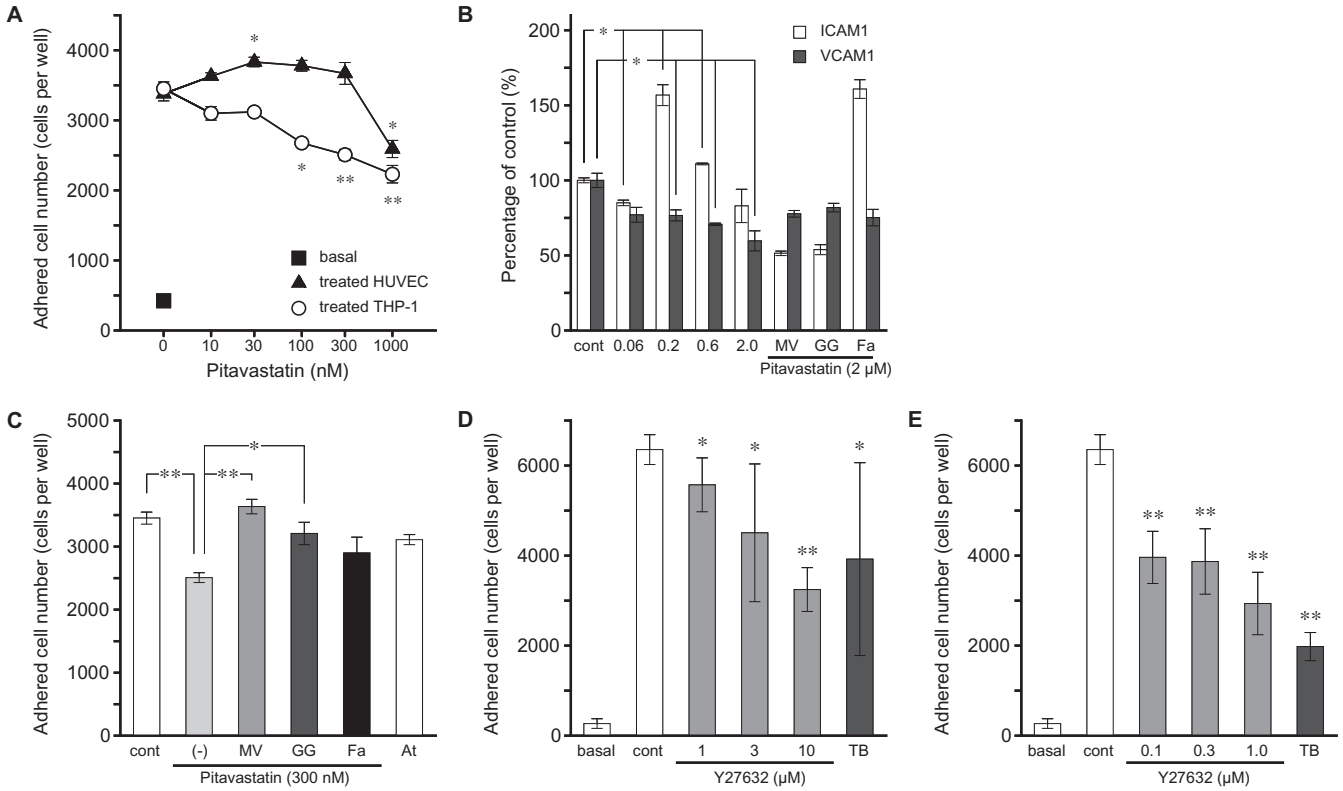

Figure 5 Effect of pitavastatin on THP-1 monocyte adhesion to human umbilical vein endothelial cells (HUVECs). (A) Dose-dependent manner of pitavastatin treatment. At the confluency of HUVEC culture, THP-1 cells or HUVECs were treated with pitavastatin for 48 h. HUVECs were treated with TNF-α (10 ng·mL^{-1}) for 8 h and THP-1 cells were labelled with BCECF-AM. THP-1 cells (2×10^5cells per well) were loaded on HUVECs and incubated for 2 h. The square at the lower left of the Figure represents the number of untreated THP-1 cells adherent to untreated HUVEC. (B) Effect of pitavastatin on cell surface expression of ICAM-1 and VCAM-1 in HUVECs. HUVECs were treated with pitavastatin alone or combined with mevalonic acid lactone (MV, 100 μM), geranylgeraniol (GG, 15 μM) or farnesol (Fa, 15 μM) for 48 h. (C) Effect of MV, GG and Fa on THP-1 cell adhesion. HUVECs were treated with pitavastatin (300 nM) alone or combined with MV (100 μM), GG (15 μM) or Fa (15 μM) for 48 h. (D) Adhesion of THP-1 cells to HUVECs treated with Y-27632. HUVECs were treated with Y-27632 for 1 h before TNF-α treatment. (E) Adhesion of THP-1 cells treated with Y-27632 to HUVECs. THP-1 cells were treated with Y-27632 for 6 h before adhesion assays. Basal, number of THP-1 cells adherent to non-treated HUVEC; cont, control; At, atorvastatin (600 nM); TB, toxin B (0.15 μg·mL^{-1}). Each column or point represents mean ± SD from a triplicate set of culture wells. *$P < 0.05$; **$P < 0.01$, significant difference from control (Student's *t*-test). HUVECs, human umbilical vein endothelial cells.

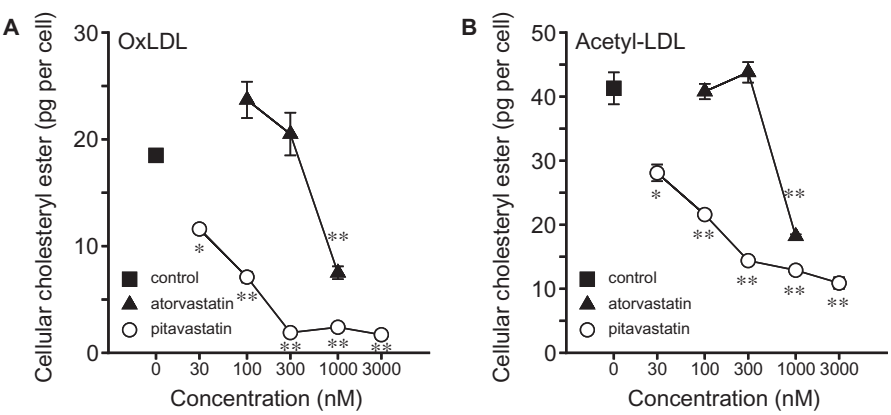

Figure 6 Effect of pitavastatin on cholesteryl ester content of RAW264.7 macrophages. RAW264.7 cells were treated with pitavastatin or atorvastatin for 48 h, then cells were treated with oxidized human plasma LDL (A) or acetylated human plasma LDL (B) for 48 h. Preparation of oxidized LDL and acetylated LDL is described in the *Methods*. The squares on the left of the Figures represent the levels of cholesteryl ester in RAW264.7 macrophages, without statin, on incubation with oxidized LDL (A) or acetylated LDL (B). Each point represents mean ± SD from a triplicate set of culture wells. *$P < 0.05$; **$P < 0.01$, significant difference from control [or pitavastatin (–) (Dunnett's test)]. LDL, low-density lipoprotein.

Table 2 Effect of mevalonic acid lactone (mevalonolactone), geranylgeraniol or farnesol on the inhibition by pitavastatin of the accumulation of cholesteryl esters in RAW macrophages

	Cellular cholesteryl ester (pg per cell)	
	Oxidized LDL	*Acetylated LDL*
Control	18.5 ± 0.6 *	41.3 ± 2.5 *
Pitavastatin (300 nM) + none	1.7 ± 0.4	10.9 ± 0.9
Pitavastatin (300 nM) + mevalonolactone 50 μM	23.0 ± 1.2 *	48.1 ± 2.2 *
Pitavastatin (300 nM) + geranylgeraniol 15 μM	3.6 ± 0.3	17.5 ± 0.1
Pitavastatin (300 nM) + farnesol 15 μM	9.9 ± 1.2	17.0 ± 0.8

*$P < 0.01$ significantly different from control; Dunnett's test.
Treatment of RAW264.7 cells and measurement of lipids were carried out as described in Figure 6. Mevalonic acid lactone (mevalonolactone), geranylgeraniol or farnesol was added in combination with 300 nM pitavastatin.
LDL, low-density lipoprotein.

inhibition of NOS suppresses hepatic CPT activity, and that it may enhance hepatic TG synthesis, VLDL secretion and intestinal MTP activity in rabbits. They also suggest that the action of pitavastatin overcomes the effects of L-NAME on serum TG.

Pitavastatin prevented intimal thickening and the accumulation of lipids and macrophages in our model. Statins have been reported to enhance expression of endothelial nitric oxide synthase (eNOS) mRNA via inhibition of Rho activity in HUVECs (Hernandez-Perera *et al.*, 1998; Martínez-González *et al.*, 2001) and eNOS expression in endothelial cells (Sumi *et al.*, 2001; Morikawa *et al.*, 2002; Terata *et al.*, 2003). In our atherosclerotic model, in the presence of chronic inhibition of NOS, pitavastatin administration may enhance eNOS expression in the arterial endothelium and thereby attenuate the effects of L-NAME on the vessel wall. Nevertheless, it remains to be clarified if pitavastatin enhances eNOS expression and affects vascular tone in rabbits treated with L-NAME. Blood pressure in the rabbits under anesthesia was not affected by administration of L-NAME and pitavastatin (data not shown).

Pitavastatin (10 μM) inhibited the contractions of aortic rings to acetylcholine *in vitro* by 20%, but less than 3 μM pitavastatin was without effect (data not shown). Because the blood concentration of pitavastatin in our treated rabbits is expected to be at nM levels (see Kojima *et al.*, 1999; Fujino *et al.*, 2002a,b), the contractile properties of the aorta might not be affected in the present study. So, we have assumed that pitavastatin did not influence blood pressure in this study.

Chronic inhibition of NOS causes increased expression of adhesion molecules, leading to enhanced monocyte attachment to the endothelium (Cayatte *et al.*, 1994). Statins reduce the adhesion of human monocytes to endothelial cells using HUVECs, U937, THP-1 and human CD14(+) monocytes (Gerszten *et al.*, 1999; Teupser *et al.*, 2001; Yoshida *et al.*, 2001; Kojima *et al.*, 2007). The number of adherent THP-1 cells decreased after treatment with pitavastatin, but this number did not change when HUVECs, rather than the THP-1 cells, were treated with pitavastatin. Reduction of macrophage accumulation in the atheromatous lesion by pitavastatin may be due to inhibition of monocyte adhesion to the endothelium, and this inhibitory effect on monocyte adhesion to the endothelium could be due to its effect on the adherence of monocytes. Attenuation of the effects of pitavastatin by mevalonic acid lactone and geranylgeraniol suggests that its inhibitory effects on adhesion of THP-1 cells may be mediated by inhibition of the activity of HMG-CoA reductase and production of isoprenoids. There are several reports that statins inhibit adhesion of monocytes to endothelial cells by inhibiting expression of adhesion molecules on the monocytes and endothelial cells by isoprenoid production and inactivation of Rho G protein (Niwa *et al.*, 1996; Serrano *et al.*, 2001; Bernot *et al.*, 2003). The effects of Y-27632 and Toxin B on THP-1 cell adhesion to endothelial cells suggest that Rho/Rho kinase signaling pathways may also play a role in monocyte/endothelial cell adhesion, and that these pathways may be suppressed by pitavastatin. These findings suggest that pitavastatin may decrease cell-surface expression of adhesion molecules on circulating mononuclear cells and endothelial cells in hypercholesterolaemia via inactivation of Rho G protein. In support of this hypothesis, statins have been reported to reduce the expression of adhesion molecules in circulating monocytes and endothelial cells in hypercholesterolaemic patients (Rezaie-Majd *et al.*, 2003; Stulc *et al.*, 2003).

Decrease of THP-1 cell adhesion to HUVECs by pitavastatin may be due to down regulation of cell surface expression of ICAM-1 and VCAM-1 in endothelial cells. Statins have been reported to suppress cell surface expression of ICAM-1 and VCAM-1 in HUVECs (Bernot *et al.*, 2003; Downar DZ *et al.*, 2004; Landsberger *et al.*, 2007)

Pitavastatin inhibited deposition of cholesteryl ester in RAW264.7 macrophages treated with acetylated LDL and oxidized LDL. Pitavastatin was more effective at inhibiting oxidized LDL-induced cholesteryl ester accumulation than that induced by acetylated LDL. Lovastatin has been reported to suppress the scavenger receptor type AI (SR-AI) and CD36 mRNA in human monocyte-derived macrophages (Pietsch *et al.*, 1996; Umetani *et al.*, 1996), so pitavastatin may also potently suppress CD36 mRNA rather than SR-AI mRNA in RAW264.7 macrophages. Pitavastatin suppressed the expression level of CD36 mRNA and SR-AI mRNA of RAW macrophages in our study (data not shown). This data was almost the same as the previous reports. Addition of mevalonic acid lactone prevented the effects of pitavastatin (Table 2), suggesting that the greater effect of pitavastatin in preventing cholesteryl ester accumulation may be due to its higher efficiency in inhibiting HMG-CoA reductase in RAW264.7 cells. Addition of geranylgeraniol minimally blocked the inhibitory effects of pitavastatin, indicating that pitavastatin may affect

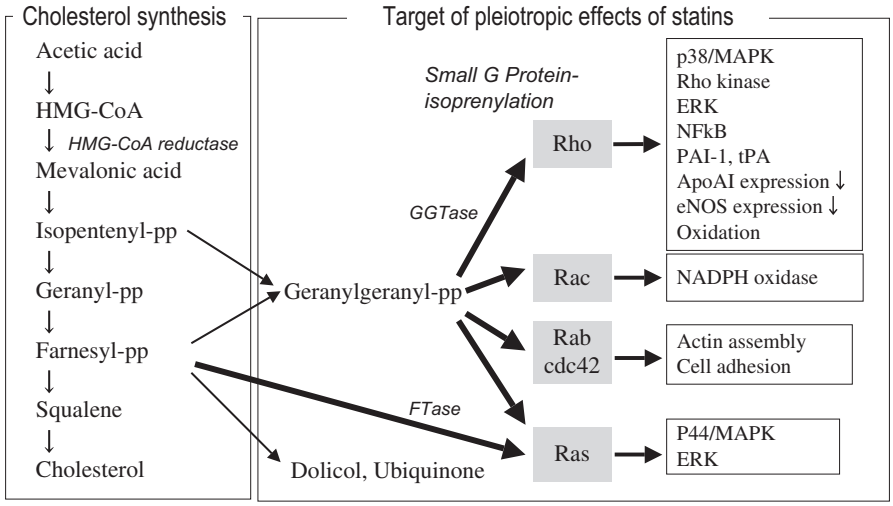

Figure 7 The mevalonate pathway and the pleiotropic effects of statins. Statins exert pleiotropic effects, via the inhibition of HMG-CoA reductase. This figure was summarized from previous reports (Endres, 2005; Alegret and Silvestre, 2006; Konstantinopoulos *et al.*, 2007). Mevalonate, a product of HMG-CoA reductase, produces several isoprenoids in the cholesterol synthesis pathway. These isoprenoids associate with proteins and modify their functions. Geranylgeranylpyrophosphate (geranylgeranyl-pp), an isoprenoid derived from cholesterol synthesis, is formed from isopentenylpyrophosphate (isopentenyl-pp) and farnesylpyrophosphate (farnesyl-pp). Geranylgeranyl-pp and farnesyl-pp are associated with the C-terminal motif of several small G proteins such as those in the Rho, Rac, Rab and Ras families, and regulate their functions. This pathway is called the 'mevalonate pathway' and plays significant roles in the biological functions of cells in several organs. Depletion of mevalonic acid via inhibition of HMG-CoA reductase by statins leads to depletion of geranyl-pp and farnesyl-pp. Depletion of geranyl-pp in turn decreases the synthesis of gernylgeranyl-pp. Finally, depletion of farnesyl-pp and geranylgeranyl-pp decrease the isoprenylation of small G proteins and thus decrease their activation and their functions. HMG-CoA, developed 3-hydroxyl-3methylglutaryl coenzyme A; GGTase, geranylgeranyltransferase I; FTase, farnesyltransferase.

an isoprenoid-independent pathway in RAW264.7 macrophages. Farnesol addition more effectively blocked the inhibitory effects of pitavastatin in oxidized LDL treated RAW264.7 macrophages, compared with geranylgeraniol. In vascular smooth muscle cells, cell migration induced by oxidized LDL was more dependent on farnesylpyrophosphate than geranylgeranylpyrophosphate (Mahadevana *et al.*, 2006). Taken together, these results suggest that farnesylated G proteins may be more relevant than geranylgeranylated G proteins, to cholesteryl ester deposition in macrophages stimulated by oxidized LDL (see Figure 7). We will publish details about isoprenoid-independent pathways in the effect of pitavastatin on formation of macrophage foam cells in the near future.

Pitavastatin also suppressed neointimal thickening in balloon-injured carotid arteries in rabbits (Kitahara *et al.*, 1998). Statins inhibit angiotensin II-mediated vascular remodelling via an anti-inflammatory effect independent of cholesterol lowering, and pitavastatin has been reported to inhibit up-regulation of intermediate conductance calcium-activated potassium channels and coronary arteriolar remodelling induced by chronic inhibition of NOS (Morikawa *et al.*, 2002). Suppression of neointimal thickening by pitavastatin is dependent on the prevention of proliferation of intimal and medial smooth muscle cells (Kitahara *et al.*, 1998), but the effective concentration of pitavastatin on the proliferation of smooth muscle cells was 10- to 100-fold higher than that for macrophage function.

In the present study, the inhibitory effects of pitavastatin on THP-1 cell adhesion and RAW264.7 macrophage foam cell formation occurred at clinically relevant concentrations. This was too low to show suppression of endothelial cells or smooth muscle cells, suggesting that these doses show selectivity between monocytes/macrophages and other cell types. Our results show that pitavastatin may predominantly suppress the invasion and subsequent accumulation of monocytes in the sub-endothelium and that pitavastatin may potently suppress lipid accumulation in macrophages. These potent monocyte-selective effects may allow pitavastatin to contribute to plaque stabilization and prevention of atherosclerosis.

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Conflicts of interest

Dr Kitahara and Dr Kanaki are employees of Nissan Chemical Industries Ltd. that produces and sells pitavastatin.

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