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Statins Alter Smooth Muscle Cell Accumulation and Collagen Content in Established Atheroma of Watanabe Heritable Hyperlipidemic Rabbits

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Background—Acute coronary syndromes often result from rupture of vulnerable plaques. The collagen content of plaques probably regulates their stability. This study tested whether HMG-CoA reductase inhibitors (statins) alter interstitial collagen gene expression or matrix metalloproteinase (MMP) levels in rabbit atheroma.

Methods and Results—We administered equihypocholesterolemic doses of pravastatin (a hydrophilic statin, 50 mg·kg⁻¹ ·d⁻¹, n=9), fluvastatin (a cell-permeant lipophilic statin, 20 mg·kg⁻¹ ·d⁻¹, n=10), or placebo (n=10) to mature Watanabe heritable hyperlipidemic rabbits for 52 weeks. The fluvastatin group achieved a much higher peak plasma concentration (23.7 μmol/L) than did the pravastatin group (1.3 μmol/L) under these conditions. Immunohistochemistry revealed that MMP-1, MMP-3, and MMP-9 expression by macrophages in the intima was lower in both the pravastatin and fluvastatin groups than in the placebo group, whereas there was no difference in macrophage numbers. Numbers of intimal smooth muscle cells (SMCs) (identified by immunohistochemistry) and expression of type I procollagen mRNA (detected by in situ hybridization), however, were significantly higher in the pravastatin group than in the fluvastatin group. Treatment with pravastatin, but not fluvastatin, preserved interstitial collagen content in vivo (detected by picrosirius red polarization). In vitro, fluvastatin, but not pravastatin, decreased numbers of rabbit and human aortic SMCs without altering procollagen I mRNA expression.

Conclusions—This study showed that statins can reduce MMP expression in atheroma and that cell-permeant statins can decrease SMC number and collagen gene expression in vivo. (Circulation. 2001;103:993-999.)

Key Words: collagen • muscle, smooth • atherosclerosis

Atherosomatous plaque rupture causes the acute coronary syndromes including unstable angina and acute myocardial infarction.¹-³ Pathological studies have shown that ruptured plaques contain a large lipid core underlying a thin fibrous cap poor in smooth muscle cells (SMCs) and collagen.⁴-⁵ Recent clinical trials have shown that lipid lowering by diet¹⁰ or cerivastatin¹¹ in atherosclerotic rabbits reduces expression of matrix-degrading proteinases by lesional macrophages and increases accumulation of interstitial collagen in atheroma, suggesting that reduced activity of proteinases permits accumulation of extracellular matrix macromolecules.¹⁰ The effect of lipid lowering on collagen gene expression by SMCs in atheroma, however, has not been determined. Recent in vitro data indicated that certain lipophilic, but not hydrophilic, statins directly induce SMC apoptosis.¹² Because of reduced collagen production, the death of SMCs might substantially impair the strength of the plaque. This study aimed to examine the regulation of collagen gene expression during lipid lowering in vivo. We also evaluated differential effects of lipophilic and hydrophilic statins on collagen synthesis by SMCs in vitro and in

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atheroma of Watanabe heritable hyperlipidemic (WHHL) rabbits, animals with endogenous hypercholesterolemia.

Methods

Animal Preparation

Twenty-nine male WHHL rabbits (>3.0 kg) were individually housed in air-conditioned rooms equipped with laminar-flow filters and fed a standard rabbit chow for 8 to 9 months. At 10 months of age, 9 rabbits started consuming pravastatin (a hydrophilic statin, 20 mg/kg), 9 rabbits started consuming fluvastatin (a lipophilic statin, 50 mg·kg⁻¹·d⁻¹), and 10 animals started consuming placebo (0.5% carboxymethylcellulose sodium). Rabbits require similar to that produced by clinically used doses of statins in humans. Pravastatin, fluvastatin, and placebo were provided by Sankyo Co Ltd (Tokyo). This study ended after 52 weeks of treatment. All animal experiments were conducted according to the guidelines for animal experimentation at the Kobe University School of Medicine.

Plasma Cholesterol and Triglyceride Levels

Peripheral blood was collected from the ear artery under local anesthesia, and plasma cholesterol and triglyceride concentrations were measured by an automated analyzer (Type 7170, Hitachi).

Figure 1. A, Macrophages and MMP localization in atheroma of WHHL rabbits. Left, Control group. Intima of WHHL rabbits of control group contains numerous macrophages detected by RAM11. Most macrophages stained positively for MMP-1 (collagenase-1), MMP-3 (stromelysin-1), and MMP-9 (gelatinase-B). Middle, Pravastatin group. Right, Fluvastatin group. Fewer than half of macrophages are MMP-1⁺, MMP-3⁺, and MMP-9⁺. Arrowheads indicate internal elastic lamina. Magnification ×100. Scale bar=200 μm. B through D. Quantitative analysis for MMP-1, MMP-3, or MMP-9 expression by macrophages. Data are reported as percentage of MMP-1⁺, MMP-3⁺, or MMP-9⁺ area within macrophage-containing regions by computer analysis. MMP-1 expression by macrophages was significantly reduced in fluvastatin group compared with control group. MMP-3 and MMP-9 expression by macrophages was significantly reduced in pravastatin and fluvastatin groups compared with control group. Probability values are determined by 1-way ANOVA followed by post hoc test. Bars represent SEM. M₀ indicates macrophages.

Figure 2. A, Localization of SMCs in atheroma of WHHL rabbits of control, pravastatin, and fluvastatin groups. Left, Control group contains numerous macrophages detected by RAM11. Most macrophages stained positively for MMP-1 (collagenase-1), MMP-3 (stromelysin-1), and MMP-9 (gelatinase-B). Middle, Pravastatin group. Right, Fluvastatin group. Fewer than half of macrophages are MMP-1⁺, MMP-3⁺, and MMP-9⁺. Arrowheads indicate internal elastic lamina. Magnification ×100. Scale bar=200 μm. B and C. Quantitative analysis for α-smooth muscle actin⁺ cells and areas within intima of atheroma. Pravastatin group has significantly more α-smooth muscle actin⁺ cells or areas in intima than did control and fluvastatin groups. Probability values are determined by 1-way ANOVA followed by post hoc test. Bars represent SEM.

Tissue Preparation

Rabbits were euthanized by administration of intravenous sodium pentobarbital (25 mg/kg). Heparin (40 U/kg) was injected simultaneously to avoid blood clotting. The aortas were excised and rinsed briefly with DMEM (BioWhittaker) without serum. The distal portion of the aortic arch (2 mm above the ligamentum arteriosum) was excised and fixed with 4% paraformaldehyde for in situ hybridization for procollagen I mRNA; immunohistochemistry for macrophages, matrix metalloproteinase (MMP)-1, MMP-3, and MMP-9; picrosirius red staining; and terminal deoxynucleotidyl transferase (TdT)–mediated dUTP-biotin nick end-labeling (TUNEL) staining. These tissues were embedded in paraffin by conventional procedures and sectioned in 5-μm slices.

In Situ Hybridization

Four oligonucleotide probes corresponding to rabbit mRNA for α₁ type I collagen₁ were synthesized: for antisense probes, 5'-ACCTTGG-GTACCTTGAAG-G-3', 5'-CCGTTTGTCCTCCTTTATGC-3', 5'-CATAGACCTTTGGGCGAG-3', and 5'-GACATGCTTTCTGCATTTG-3'; for corresponding sense probes, 5'-CTTCTAGGGTGAGGAACTC-3', 5'-GCATTAAGGGACACACGGGC-3', 5'-CTACAGCCCAAGGACTATG-3', and 5'-CAAGGCAAGGACATGC-3', labeled with 5'-fluorescein and purified by high-performance liquid chromatography (Integrated DNA Technologies, Inc). The specificity of these oligonucleotides was confirmed by sequencing of reverse transcription–polymerase chain reaction (RT-PCR) products.

In situ hybridization was performed with the Hy-Probe Detection System (Shandon/Lipshaw) as follows. After deparaffinization, slides were treated with proteinase K for 8 minutes and then dehydrated. The slides were incubated for 10 minutes at 65°C and then for 2 hours at 37°C in a humid chamber with 1 ng/μL of the oligonucleotides in hybridization buffer composed of 50% formamide, 0.6 mol/L NaCl, 10% dextran sulfate, 50 mmol/L Tris pH 7.5, 0.1% sodium pyrophosphate, Denhardt’s solution, and 5 mmol/L EDTA. After incubation, slides were washed with PBS (pH 7.4) containing 0.1% Triton X-100 and incubated with blocking solution for 10 minutes. Slides were then incubated with alkaline phosphatase–conjugated detection antibody against fluorescein for 30 minutes. Alkaline phosphatase activity was revealed by NBT/BCIP chromogen solution. Slides were counterstained with methyl green and mounted.

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by explant outgrowth were seeded in 6-well dishes for determinations of cell numbers and in flasks (150 cm²) for RNA extraction. They were incubated with DMEM supplemented with 10% FCS. Twenty-four hours later, the medium was changed to DMEM with 0.4% FCS, and the cells were incubated for 48 hours. The medium was then replaced with DMEM containing 10% FCS in the presence of absence of statins, and the cells were incubated for an additional 72 hours at 37°C. Cell number was evaluated by CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS assay, Promega).

Reverse Transcription–Polymerase Chain Reaction
RNA was isolated from arterial SMCs with RNaseasy and RNase-Free DNase (Qiagen). Then RT-PCR was performed with Superscript II RNase H–reverse transcriptase. Oligonucleotide probes were synthesized for procollagen I mRNA: 5′-site, 5′-CATAGTCTCTGAGTGGTCTGAG-3′; 3′-site, 5′-GCATAAAGGGA-CACACCGG-3′.13 For GAPDH (internal control): 5′-site, 5′-CGATGCTGTTGGAATC-3′; 3′-site, 5′-AGGGATGTAGTT-CTGGGC-3′. Polymerase chain reaction amplification used the following conditions: denaturation for 1 minute at 94°C, annealing for 1 minute at 55°C, and elongation for 1 minute at 72°C for 25 cycles. RNA levels were normalized by amplification of GAPDH.

Quantitative Analysis for Histology and Statistics
Quantification of immunohistochemistry for macrophages, MMP-1, MMP-3, MMP-9, α-smooth muscle actin, and Sirius red polarization was performed by computer-assisted color image analysis (Image Pro Plus). We divided MMP-1+, MMP-3+, and MMP-9–positive areas within macrophage-containing regions by the whole macrophage area, denoted MMP-1(+)/macrophages, MMP-3(+)/macrophages, and MMP-9(+)/macrophages. The positive areas were calculated in Excel (Microsoft). Analyses of in situ hybridization for procollagen I mRNA, immunohistochemistry for α-smooth muscle actin, and TUNEL–positive cells within SMC-containing regions were performed manually by 2 different observers who counted all positive cells in a blinded manner. Interobserver correlation was excellent (r=0.98). Statistical testing used 1-way ANOVA followed by the post hoc test.

Results
Plasma Lipids and Concentrations of Statins
At the beginning of the experiment, there was no significant difference in total cholesterol level among the 3 groups and after 52 weeks of treatment: 421±29, 315±43, and 332±22 mg/dL for the control, pravastatin, and fluvastatin groups, respectively. The pravastatin and fluvastatin groups had significantly lower total exposure to cholesterol than the control group, however, as measured by the area under the curve (AUC): 29 149±1559, 21 167±1947, and 21 783±1462 mg·wk⁻¹·dl⁻¹ for the control, pravastatin, and fluvastatin groups, respectively. The pravastatin and fluvastatin groups had similar decreases in AUC, indicating that these 2 statins had equihippocholesterolemic effects in these WHHL rabbits at the doses used.

Under these conditions, fluvastatin achieved much higher blood levels than did pravastatin (C_max, 1.29±0.28 and 23.70±2.75 μmol/L; AUC, 11.9±2.7 and 124.0±11.8 μmol·L⁻¹·h⁻¹ for the pravastatin and fluvastatin groups, respectively, *P<0.01). Statins Reduced MMP-1, MMP-3, and MMP-9 Protein Expression by Macrophages
There was no significant difference in the intimal cross-sectional area or the macrophage-containing area in the control, pravastatin, and fluvastatin groups (intimal area, 5.59±0.61, 5.44±0.55, and 5.64±0.40 mm², P=0.58; RAM-11–positive area,
0.62±0.34, 0.71±0.20, and 0.88±0.38 mm², P=0.30 by ANOVA, for the control, pravastatin, and fluvastatin groups, respectively. There was no signal in negative controls.

We further quantified MMP-1–, MMP-3–, and MMP-9– positive areas within macrophage-containing regions (Figure 1A). The fluvastatin group had significantly less MMP-1 positivity within macrophage areas (Figure 1B), and the pravastatin and fluvastatin groups had significantly less MMP-3– or MMP-9–positive areas within the macrophage areas compared with the control group (Figure 1C and 1D).

**Pravastatin Treatment Produced More Smooth Muscle Accumulation and Higher Procollagen I mRNA Levels**

Immunohistochemistry for α-smooth muscle actin showed that the areas and numbers of immunopositive cells were higher in the atheroma of WHHL rabbits in the pravastatin group than in the fluvastatin and control groups (Figure 2). Procollagen I mRNA expression (detected by in situ hybridization) was also significantly higher in the pravastatin than in the fluvastatin or the control group (Figure 3). There was no significant difference in the ratio of procollagen I mRNA expression to α-actin–positive cells among the 3 groups (Figure 3C and 3D). These results suggest that the increased procollagen I mRNA levels in the pravastatin group resulted from an increased number of SMCs.

**Pravastatin Increases Collagen Content**

Picrosirius red polarization showed significantly increased interstitial collagen content in the intima of WHHL rabbits in the pravastatin group compared with the fluvastatin and
control groups (Figure 4). The adventitia constitutively showed copious fibrillar collagen in all 3 groups.

**Death of SMCs**

Some in vitro studies have suggested that statins promote apoptosis of SMCs in vitro. Therefore, to examine DNA fragmentation in SMCs in atheroma of WHHL rabbits, TUNEL staining was performed. There was no significant difference in the numbers of TUNEL-positive cells in the 3 groups (Figure 5).

**Fluvastatin Inhibits SMC Growth Much More Potently Than Pravastatin but Neither Statin Affects Collagen mRNA Levels In Vitro**

In vitro experiments examined potential direct effects of pravastatin and fluvastatin on growth and procollagen I gene expression in rabbit and in human aortic SMCs. Fluvastatin, but not pravastatin, reduced growth of both rabbit and human aortic SMCs in a dose-dependent manner (Figure 6). There was no significant difference in rabbit procollagen I mRNA expression among control, pravastatin-treated, and fluvastatin-treated cells over 72 hours at 2 different doses of statins (Figure 7).

**Discussion**

Collagen content contributes critically to atherosclerotic plaque stability. The present study demonstrated that collagen accumulation in complex atheroma of WHHL rabbits correlates with the number of SMCs, a major source of extracellular matrix. This study also shows differential effects of a hydrophilic and lipophilic statin on SMC accumulation and collagen content in these lesions. Pravastatin has high selectivity for hepatocytes as a result of its hydrophilicity, in contrast with cell-permeant lipophilic statins, such as fluvastatin. Pravastatin achieved an equal degree of cholesterol lowering in WHHL rabbits in the present study at significantly lower blood concentrations than fluvastatin. In this study, the concentration of fluvastatin was some 20 times higher than that of pravastatin. These differences also apply to humans. In normal human volunteers, 20 mg of pravastatin yielded a Cmax of 86.0 nmol/L (38.4 ng/mL, Tmax 1.1 hours). Conversely, 20 mg of fluvastatin yielded a Cmax of 418 nmol/L (181 ng/mL, Tmax 0.5 to 1.5 hours).

Ruptured human atherosclerotic plaques often have a thin fibrous cap with little collagen and few SMCs. SMC loss might result from cell death due in part to apoptosis in human atheroma and in hypercholesterolemic rabbits. In fact, hypercholesterolemia reduces SMC number and collagen content and causes mechanical weakening of rabbit atheroma. Shiomi et al demonstrated that atheroma of WHHL rabbits that had undergone cholesterol reduction with pravastatin contain more SMCs and collagen accumulation than those of placebo-treated animals. Kockx et al also reported that lipid lowering decreases apoptotic vascular cells in rabbit atheroma. In the present study, the tendency toward a decreased number of TUNEL-positive cells furnished one potential mechanism by which lesions in the pravastatin group contained more SMCs and collagen than those in the control group. Thus, lipid lowering may suppress the death of SMCs and in turn stabilize the plaque.

Lipophilic statins can suppress proliferation and induce apoptosis of SMCs in vitro. Corsini et al showed that serum from patients treated with fluvastatin, but not pravastatin, inhibited the proliferation of cultured human arterial SMCs.
Buemi et al.\textsuperscript{23} also reported that serum from patients treated with fluvastatin significantly reduced proliferation and increased apoptosis in human SMCs. Guijarro et al.\textsuperscript{12} showed that lipophilic statins, such as atorvastatin, simvastatin, or lovastatin, but not hydrophilic pravastatin, induced apoptosis of rat SMCs in a dose-dependent manner. Soma et al.\textsuperscript{24} previously described an antiproliferative effect of fluvastatin, but not pravastatin, on SMCs in acute vascular injury in nonatherosclerotic normcholesterolemic rabbits. The present study showed that fluvastatin concomitantly decreased SMC and collagen accumulation in atheroma of WHHL rabbits in vivo.

Macrophage activation also plays an important role in the pathogenesis of acute coronary syndromes.\textsuperscript{4,25} Lesional macrophages produce proteolytic enzymes, including members of the MMP, cysteine protease, and plasminogen activator families. Thus, macrophage-related proteolysis within atheroma may weaken the protective fibrous cap and promote plaque rupture.\textsuperscript{26} We previously demonstrated that dietary lipid lowering reduces the number of macrophages expressing MMPs and tissue factor in rabbit atheroma.\textsuperscript{10,27} In addition to lower macrophage numbers, reduced macrophage activation could be an important therapeutic goal. Statins such as fluvastatin or simvastatin reduce MMP-9 secretion by macrophages in culture.\textsuperscript{28} The present study demonstrates that pravastatin or fluvastatin significantly reduces MMP expression but not macrophage number in vivo, which suggests decreased macrophage activation. This alteration should promote plaque stabilization.

We have hypothesized that a dynamic balance between collagen synthesis and degradation determines collagen content in the fibrous cap of the plaque. Evidence that such a balance operates in vivo and hence regulates plaque stability, however, remains scant. Lipid lowering can affect both SMC phenotype and macrophage activation. We previously demonstrated that dietary lipid lowering promotes accumulation of mature SMCs in rabbit atheroma.\textsuperscript{29} SMCs in the modulated phenotype (so-called “synthetic”-state cells) can synthesize 2- to 3-fold more collagen than mature phenotype (“contractile”-state cells) in vitro.\textsuperscript{30} Thus, lipid lowering may reduce collagen production by SMCs. Lipid lowering, however, increased the collagen content of rabbit atheroma.\textsuperscript{10} This finding may reflect a greater local decrease in proteolytic activity than in collagen synthesis. These results indicate that a marked reduction in collagen degradation can outweigh a slight decrease in collagen synthesis, leading to an increase in collagen accumulation in atheroma.

This study was not designed to test the clinical utility of the agents used but rather to test the hypothesis that statins can differentially affect variables related to plaque stability in humans in an atherosclerotic animal with very high blood levels of fluvastatin. We aimed to explore in vivo the controversial notion of “pleiotropic” effects of statins. Indeed, our results establish a distinct difference in in vivo effects of 2 statins on spontaneous atheroma in WHHL rabbits. These results should not be extrapolated directly to the use of these 2 agents in humans, however, because the levels of fluvastatin in this experiment exceed those expected in humans. Nonetheless, these results strongly support the concept that statins can have different in vivo effects on SMC accumulation and collagen production independent of cholesterol lowering. Our findings further provide important information for development of more effective therapies for atherosclerosis.

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