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Statin prevents plaque disruption in apoE-knockout mouse model through pleiotropic effect on acute inflammation

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ABSTRACT

Although it has been demonstrated that statins stabilize atherosclerotic lesions in animal models of advanced atherosclerosis, there is little evidence to suggest that statins have a preventive effect on plaque rupture itself. In the present study, we examined the effect of fluvastatin on plaque disruption using a simple and quick method of plaque disruption in carotid artery lesions in apolipoprotein E-deficient mice. Male apolipoprotein E-deficient mice received normal chow and underwent ligation of the left common carotid artery just proximal to its bifurcation. Four weeks later, a polyethylene cuff was placed around the artery immediately proximal to the ligation site. Fluvastatin (10 mg/kg per day) was given by oral gavage every day starting at 3 days before cuff placement. The administration of fluvastatin suppressed atherosclerotic plaque disruption accompanied by luminal thrombi by 31.5% compared with controls at 4 days after the cuff was placed at the ligated carotid artery. Fluvastatin administration decreased matrix metalloproteinase-9 expression, gelatinolytic activity, endothelial adhesion molecules expression and neutrophil infiltration, and increased type I collagen content in the cuffed region. In summary, fluvastatin was found to prevent plaque disruption through pleiotropic effect on acute inflammation in an animal model using apolipoprotein E-deficient mice.

1. Introduction

Lipid lowering with statins, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, has been established as an important therapy in the primary and secondary prevention of atherosclerotic disease [1,2]. However, in some clinical trials, statins have been found to reduce cardiovascular morbidity and mortality to a degree beyond what is expected from reductions in low-density lipoprotein cholesterol (LDL-C) levels alone [3,4], raising the possibility of clinically important effects beyond LDL-C reduction on cardiovascular disease [5]. In addition, recent studies have raised the additional possibility of positive short-term effects when statins are given to treat acute coronary syndrome (ACS) [6,7]. The significant benefits associated with the early initiation of statin treatment in ACS have added to the growing evidence supporting the hypothesis of pleiotropic effects of statins.

Plaque formation in the coronary arteries or plaque rupture in the peripheral vasculature in the later stages of atherosclerosis triggers the onset of acute ischemic events including myocardial and cerebral infarction. Although several plaque rupture models have been proposed, plaque rupture has been seen infrequently, even in old high serum cholesterol-prone mice after prolonged feeding with very high-cholesterol diets [8,9]. This means that intervention studies must necessarily involve weeks or even months of treatment, which is problematic in the case of agents that are scarce and/or expensive. Recently, we provided a novel, simple, fast and highly efficient model for the progress of atherosclerotic plaque vulnerability and disruption in apolipoprotein E-deficient (apoE-deficient) mice [10]. Although it has been demonstrated that statins stabilize atherosclerotic lesions in animal models of advanced atherosclerosis [11–13], there is little evidence to date that suggests that statins have a preventive effect on plaque disruption itself, with the exception of the report demonstrating plaque-stabilizing effects of pravastatin at brachiocephalic in apoE-deficient mice by Johnson et al. [14].

In the present study, we examined whether fluvastatin has a beneficial effect on plaque disruption by use of our novel mouse model.

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2. Materials and methods

2.1. Animals

All animal experiments were performed in accordance with the animal care guidelines of Nagoya University Graduate School of Medicine. Our experimental animals were 8-week-old apoE-deficient mice (C57BL/6) weighing between 21 and 25 g and purchased from Jackson Laboratory. The mice were provided with a standard diet (Oriental Yeast) and tap water ad libitum throughout the experimental period. Surgery was performed essentially as previously described [10]. In brief, the animals were anesthetized with an intraperitoneal (i.p.) injection of pentobarbital sodium (50 mg/kg; Dainippon Pharmaceutical). Ligation of the left common carotid artery in 9-week-old mice was performed just proximal to the bifurcation, following the procedure described previously. Four weeks after ligation, a polyethylene cuff was applied just proximal to the ligated site.

2.2. Experimental design and tissue collection

The mice were randomly assigned to one of two groups: the control group (vehicle; 0.5% carboxymethylcellulose) and the fluvastatin group (10 mg/kg per day). Dose of fluvastatin was referred to the previous report [15]. The treatments were given by oral gavage every day starting at 3 days before cuff placement. At various time points, mice were anesthetized by i.p. pentobarbital injection and blood samples were collected in heparinized syringes. The carotid arteries in the cuff were harvested for analysis by several methods and were processed as described below. Fluvastatin was supplied by Novartis Pharma AG.

2.3. Histological and immunohistochemical staining

Just before cuff placement (day 0) and 2 or 4 days after cuff placement, mice were perfused through the left cardiac ventricle with isotonic saline and 4% paraformaldehyde in 0.01 M phosphate buffer (pH 7.4) under physiological pressure. Carotid arteries were collected and processed for histological analysis as described previously [16]. Cross-cryosections (6 μm) were prepared from the intracuff region of each carotid artery and stained routinely with hematoxylin and eosin (H&E), and picrosirius red for collagen. The corresponding sections on separate slides were used for immunohistochemical staining. The sections were preincubated with 5% serum and then incubated with antibodies against neutrophils (1:50; Serotec) and matrix metalloproteinase (MMP)-9 (1:100; BIOMOL International LP). Immunohistochemical staining was visualized using an ABC Kit (Vector Laboratories) following the manufacturer's instructions. Levamisole (Vector Laboratories) was used as the inhibitor of endogenous alkaline phosphatase. Counterstaining for the nucleus was performed with Mayer's hematoxylin.

2.4. Morphometric analysis

All morphometric analyses were made on H&E stained sections. Histological classification of the plaque disruption at the intracuff region of the carotid artery was done according to previously published methods [11]. Briefly, the lesions were divided into three groups, based on the analyses of 30 sections at 60-μm intervals in each sample tissue. When there were no cracks and no mural or occlusive thrombus at the intracuff region, we classified them into "no disruption". When intraplaque hemorrhage, or mural or occlusive thrombus with cracks or erosion in the plaques were detected, these were classified into "hemorrhage" or "disruption", respectively.

2.5. Determination of collagen and neutrophils content

Collagen content was evaluated by the picrosirius red-stained positive area which appears bright when viewed with polarized light. Neutrophils infiltrated in the intima were assessed by the neutrophils positive area which was stained by anti-neutrophil antibody (1:50; Serotec, MCA771GA). For the quantification of collagen content and infiltration of neutrophils in the sections, National Institutes of Health Image software was used for all images. We set a threshold to automatically compute the positive areas for each stain and then computed the ratio of positive area to the intimal area.

2.6. Double immunofluorescence

Colocalization studies were performed with double immunofluorescence staining methods. The sections were preincubated with 5% serum and incubated with antibodies against MMP-9 (1:100; BIOMOL), neutrophils, and/or macrophages (1:100; BD Pharmingen) for 18 h. Immunoreactivity was visualized using a fluorescein-conjugated anti-rabbit IgG (1:100; Vector Laboratories) or Texas Red-conjugated anti-rat IgG (1:100; Vector Laboratories). The slides were mounted in glycerol-based Vectashield medium (Vector Laboratories) containing the nucleus stain 4′,6-diamidino-2-phenylindole (DAPI).

2.7. Real-time reverse transcription-polymerase chain reaction

Just before and 1, 2 or 4 days after cuff placement, carotid arteries from the intracuff region were collected without perfusing with 4% paraformaldehyde and soaked in RNA*later* (Ambion). Total RNA was extracted using a Qiagen RNeasy Micro Kit (Qiagen, Inc.) following the manufacturer's instructions. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis using the Taqman method and the ABI 7300 real-time PCR System (Applied Biosystems) was performed as described previously [17]. The sequences of the primers and probes are shown in Table 1; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was measured in parallel with genes of interest and used as an internal standard.

2.8. In situ zymography

Gelatinolytic activity was analyzed using an MMP *in situ* Zymo-Film (Wako, Inc.). An MMP-PT *in situ* Zymo film containing 1,10-phenanthroline as an MMP inhibitor to block protease activity was used as a control. Just before and 4 days after cuff placement, tissues were collected from the intracuff region without perfusing with 4% paraformaldehyde, and were then frozen immediately in optimum cutting temperature (OCT) compound (Sakura Finetechnical). Frozen sections (4 μm) were placed on the film and incubated for 30 h at 37 °C in a moisture box following the manufacturer's instructions. The specimens were then stained with Amido black 10B (Sigma) for 15 min and destained for 10 min. The areas of gelatinolytic activity were visualized under a light microscope.

2.9. Statistical analysis

Data were represented as means ± S.E.M. One- or two-way analysis of variance (ANOVA) followed by post hoc testing (Scheffe test) or chi-squared test was used for statistical analysis where appropriate at $P < 0.05$.

Table 1
Taqman primer and probe sequences used in real-time PCR analysis.

Gene	Forward primer sequence	Reverse primer sequence	Probe sequence
Matrix metalloproteinase-2 (MMP-2)	CCCCATGAAGCCTTGTTTACC	TTGTAGGAGGTGCCCTGGAA	CAATGCTGATGGACAGCCCTGCA
Matrix metalloproteinase-3 (MMP-3)	CATGGAGACTTTGTCCCTTTTGAT	CGTCAAAGTGAGCATCTCCATTA	TGGCTCATGCCTATGCACCTGGAC
Matrix metalloproteinase-9 (MMP-9)	AGACCAAGGGTACAGCCTGTTC	GGCACGCTGGAATGATCTAAG	CGCACGAGTTCGGCCATGCAC
Matrix metalloproteinase-14 (MMP-14)	GTCAGCCTGCTTCTCATGTCC	CCACGCCACTGCGCTT	CGGATGTAGGCATAGGGCACTTCTCG
Tissue inhibitor of metalloproteinases 1 (TIMP-1)	GCCTACACCCCAGTCATGGA	GGCCCGTGATGAGAAACTCTT	TGGATATGCCCACAAGTCCCAGAACC
Tissue inhibitor of metalloproteinases 2 (TIMP-2)	GTCCCATGATCCCTTGCTACA	TGCCCATTGATGCTCTTCTCT	CTCCCCGGATGAGTGCCTCTGGA
Collagen type I	AGGCGAAGGCAACAGTCG	GTTCCGGYGTGACTCGTGC	ACCTACAGCACCCTTGTGGACGGC
Intercellular adhesion molecule 1 (ICAM-1)	CCCCGCAGGTCCAATTC	CCAGAGCGGCAGAGCAA	CACTGAATGCCAGCTCGGAGGATCAC
Vascular cell adhesion molecule 1 (VCAM-1)	ACAAAACGATCGCTCAAATCG	GGTGACTCGCAGCCCGTA	CTCCATGGCCCTCACTTGCAGCA
P-selectin	GCAAAGGCATAACATCACTTCCTG	AAAGCTTCCCAGGTGGTGTTG	AGTCCGATGCCCTGCCCTCACGA
E-selectin	TGTACGTCCTCTGGAGAGTGG	GCAGGGTGGGTCAAAGCTTC	CGCCTGCTCCAGCCTGCCATGTG

3. Results

3.1. The effect of statin on plaque disruption in carotid arteries

There were no differences in plasma total cholesterol levels between the fluvastatin and control groups (21.7 ± 1.5 mmol/L, $n = 33$ vs. 18.8 ± 1.1 mmol/L, $n = 32$; $P = 0.1$).

Before cuff placement (day 0), no significant differences in the histological findings at the equivalent sites of the cuffed region of the carotid artery were observed between the fluvastatin and control groups (Fig. 1A and B). At 4 days after cuff placement, the proportions of intraplaque hemorrhage and disruption in the neointima accompanying the intramural thrombus were compared between the fluvastatin and control groups. Fluvastatin administration was found to suppress atherosclerotic plaque disruption by 31.5% compared with controls at 4 days after cuff placement at the ligated carotid artery (Table 2, Fig. 1C and D), although no difference in the rate of intraplaque hemorrhage was detected between these two groups (Table 2).

Table 2
Fluvastatin reduces in percentage of plaque disruption in the murine model.

	n	Disruption	No disruption	
			Hemorrhage	No hemorrhage
Fluvastatin	25	24.0 (6)	36.0 (9)	40.0 (10)
Control	18	55.5 (10)	38.9 (7)	5.6 (1)

Tissue sections were classified into three groups based on our analysis of 30 sections at 60-μm intervals in each sample tissue. Disruption, presence of mural or occlusive thrombus with cracks or erosion in the plaques; hemorrhage, presence of intraplaque hemorrhage; no disruption and no hemorrhage, no cracks and no mural or occlusive thrombus. Data: %(*n*). $P < 0.05$ (chi-squared test).

3.2. Messenger RNA quantification

The levels of MMP-2, -3, -9 and -14, tissue inhibitor of metalloproteinases (TIMP)-1 and -2, type I collagen, vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, E-selectin, and P-selectin mRNAs were determined by quantitative real-time RT-PCR (Fig. 2). An increase in MMP-9 mRNA was

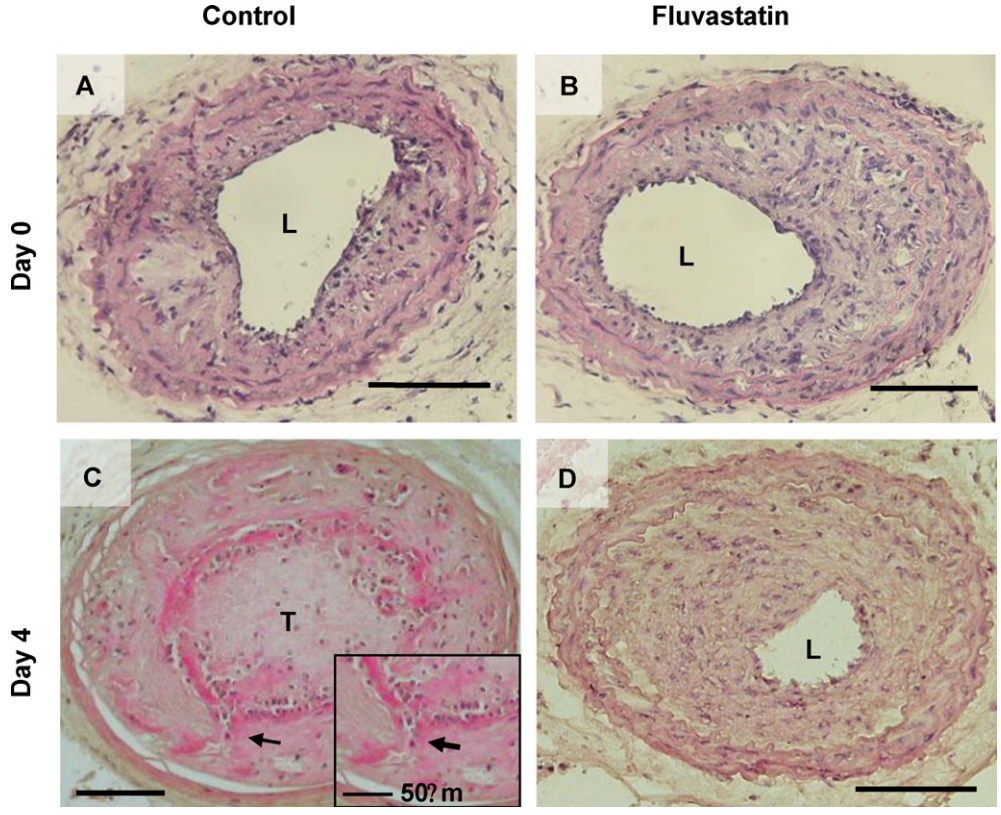

Fig. 1. The effect of fluvastatin on plaque disruption formation in the left carotid arteries of apoE-deficient mice. A through D, H&E staining of cross-sections of the left carotid artery. A and C, control; B and D, fluvastatin treatment; day 0, just before cuff placement; day 4, at days 4 after cuff placement. Bars = 100 μm. T (C) indicates thrombus, L (A and B) indicates lumen and arrow (C and inset) indicates the crack of fibrous cap.

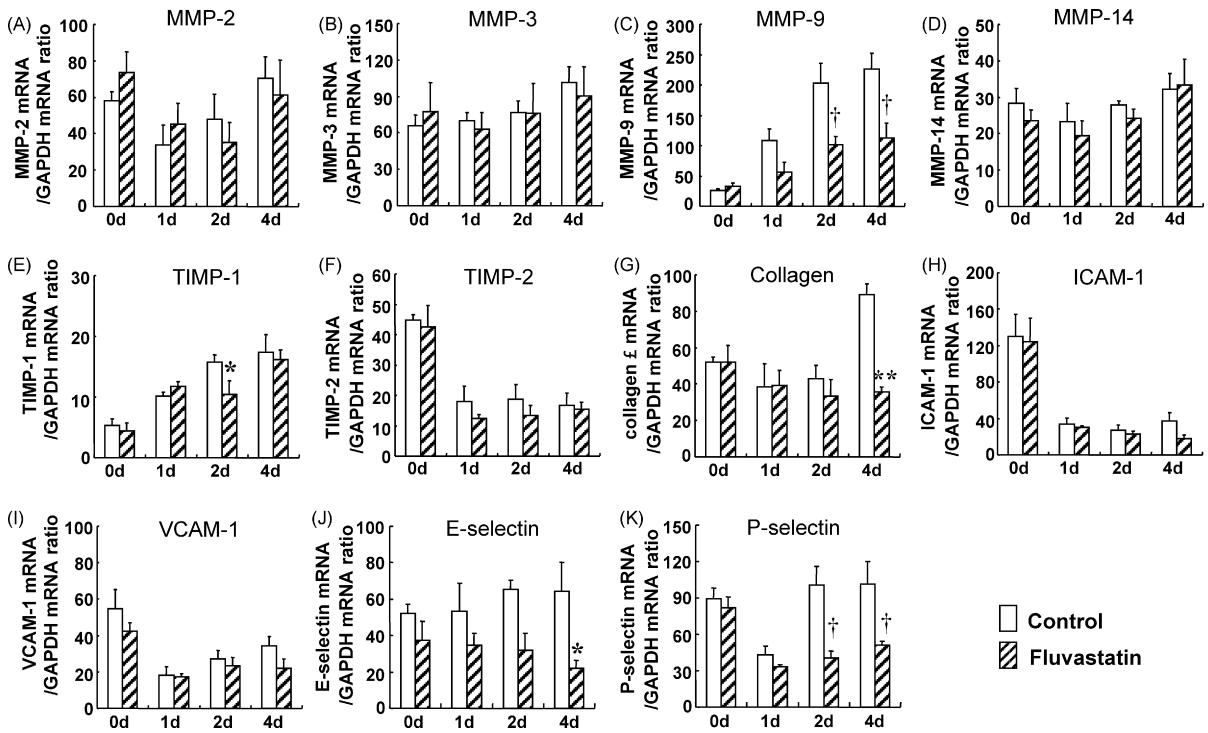

Fig. 2. Quantitative real-time RT-PCR analysis of the mRNAs for MMP-2 (A), MMP-3 (B), MMP-9 (C), MMP-14 (D), TIMP-1 (E), TIMP-2 (F), type I collagen (G), ICAM-1 (H), VCAM-1 (I), E-selectin (J), and P-selectin (K) in the left carotid arteries of fluvastatin-treated ($n = 5$) or control mice ($n = 5$) at the indicated time points. All data were normalized according to GAPDH mRNA; means ± S.E.M.; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\dagger}P < 0.001$.

observed at days 1, 2 and 4 after cuff placement in control mice, and this increase was significantly suppressed by fluvastatin at days 2 and 4 after cuff placement (Fig. 2C). The mRNA expression of MMP-2, -3 and -14 did not differ between the fluvastatin and control groups in the cuffed artery during the follow-up period after cuff placement (Fig. 2A, B and D). No significant differences were observed in TIMP-1 or -2 mRNA levels up to 4 days after cuff placement, with the exception of TIMP-1 mRNA levels at day 2. The amount of type I collagen mRNA was significantly suppressed at 4 days after cuff placement in the fluvastatin group compared with controls (Fig. 2E and G), although there was no significant difference in TIMP-2 mRNA between these groups (Fig. 2F). E-selectin and P-selectin mRNA levels were significantly suppressed by fluvastatin (Fig. 2J and K), although no statistical differences were detected in VCAM-1 and ICAM-1 mRNA levels between the fluvastatin and control groups (Fig. 2H and I).

3.3. *In situ zymography*

In situ zymographic analysis revealed gelatinolytic activity primarily in the neointimal region in control carotid arteries at 4 days after cuff placement (Fig. 3A), and this activity was suppressed in the carotid arteries of fluvastatin-treated mice (Fig. 3B). No gelatinolytic activity was observed in control specimens containing phenanthroline (Fig. 3C and D).

3.4. *Collagen content in the neointima*

There was no difference in collagen content between the fluvastatin and control groups just before cuff treatment (Fig. 3E, F and I). Although a significant decrease in collagen content was observed in control carotid arteries at day 4 compared with levels before cuff placement (Fig. 3G), the administration of fluvastatin successfully maintained the collagen content in the carotid artery after cuff placement (Fig. 3H).

3.5. *Neutrophil infiltration in the neointima*

Neutrophil infiltration was increased at days 2 and 4 after cuff placement at the ligated artery in the control group (Fig. 4A–C and E). The administration of fluvastatin was found to have significantly suppressed the neutrophil-positive area in the neointima of the carotid artery compared with controls at 4 days after cuff placement (Fig. 4D).

3.6. *MMP-9 localization*

Immunohistochemical analysis revealed that more intense MMP-9 staining was observed at the plaque region and in the atheromatous lesions in control carotid arteries at 2 days after cuff placement compared with the fluvastatin-treated group (Fig. 5A–E and H). Double labeling of sections of carotid arteries with antibodies to the macrophage- or neutrophil-specific markers (Fig. 5F and I, respectively) and MMP-9 revealed that MMP-9 was colocalized with macrophages and neutrophils (Fig. 5G and J, respectively).

4. Discussion

A number of studies have demonstrated that statins reduce MMP protein expression and activities when administered to hyperlipidemic animals, and that this is accompanied by changes in plaque morphology consistent with increased stability [11–13]. Moreover, the plaque-stabilizing effects of statins in animal models can apparently be obtained even independently of cholesterol lowering [12,13], which implies a direct effect of statins on the mechanisms leading to plaque instability. However, there is little direct evidence of the beneficial effects of statin on plaque rupture. Recently, Johnson et al. reported the interesting finding that pravastatin treatment prevents early plaque rupture in the brachiocephalic arteries of apoE-knockout mice, which demonstrated a direct beneficial effect on plaque stability [14].

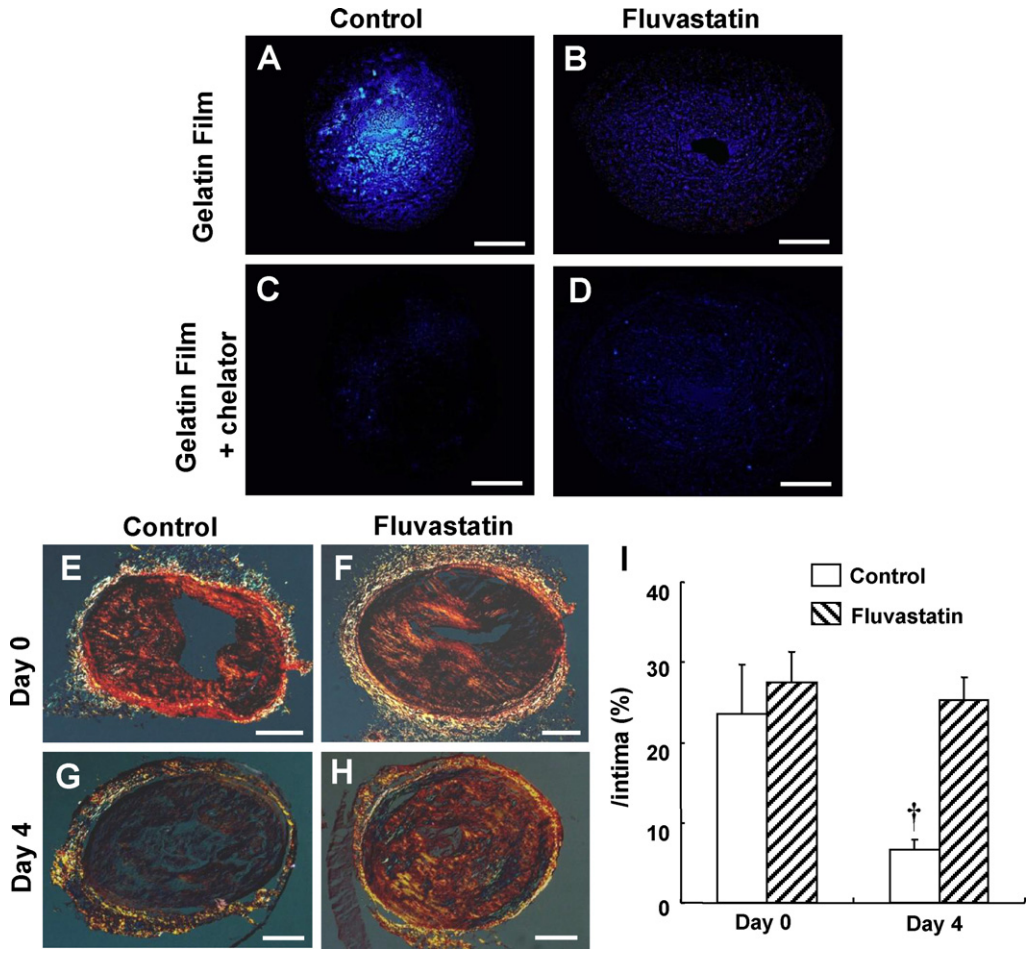

Fig. 3. Gelatinolytic activity and collagen content in the left carotid arteries in the fluvastatin and control groups. A through D, *in situ* zymography at 4 days after cuff placement. Brightness under a light microscope shows degradation with gelatinases. A and B: gelatin film; C and D: gelatin film with a chelator serving as a negative control. Bars = 100 μm. E through H, picrosirius red staining of specimens viewed under polarized light before cuff placement (day 0) and at 4 days after cuff placement. I, quantitative analysis of collagen content. Data were determined by computer analysis and were expressed as a percentage of the positive area for picrosirius red staining within the intima. Values are means ± S.E.M.; $n = 5$ (fluvastatin, day 0), $n = 16$ (fluvastatin, day 4), $n = 5$ (control, day 0), $n = 14$ (control, day 4), $^{\dagger}P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

In the present study, we observed that fluvastatin reduced the rate of plaque disruption with mural thrombosis in our mouse model. As previously demonstrated [12–14], statin use has no effect on lipid levels in apo-E-deficient mice, suggesting that this effect of fluvastatin on plaque disruption in the present model is independent of plasma cholesterol lowering. It should be noted that fluvastatin administration had no effect on the induction of intraplaque hemorrhage after cuff placement, suggesting that this type of hemorrhage may not stem from plaque disruption but from intraplaque microvessels. In fact, recent studies in human and animal model have suggested that intraplaque hemorrhage was observed in atherosclerotic plaque regions, which is thought to be

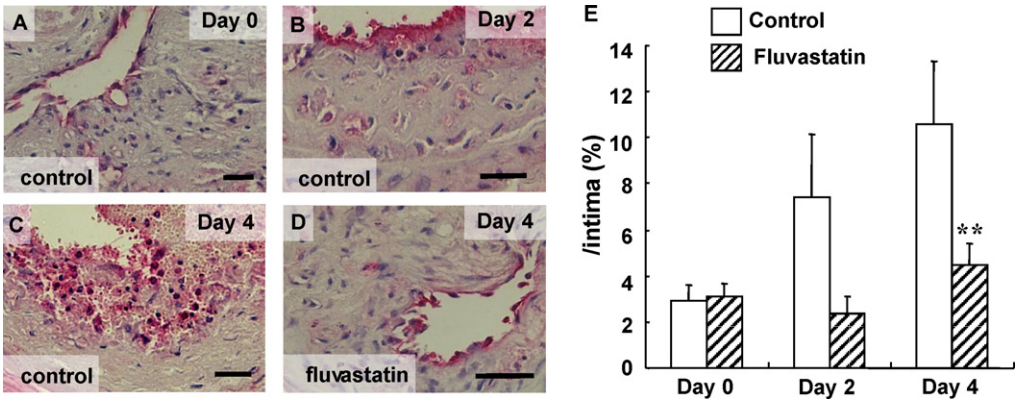

Fig. 4. Infiltration of neutrophils in the left carotid arteries before and after cuff placement. Immunoreactivity of neutrophils at day 0 (A), day 2 (B) and day 4 (C) in the control group after cuff placement, and at day 4 after cuff placement in the fluvastatin group (D). E, percentage of the neutrophil-positive area within the total intimal area. Values are means ± S.E.M. Fluvastatin group, $n = 12$ (day 0), $n = 10$ (day 2), $n = 14$ (day 4); control group, $n = 10$ (day 0), $n = 6$ (day 2), $n = 14$ (day 4), $^{**}P < 0.01$.

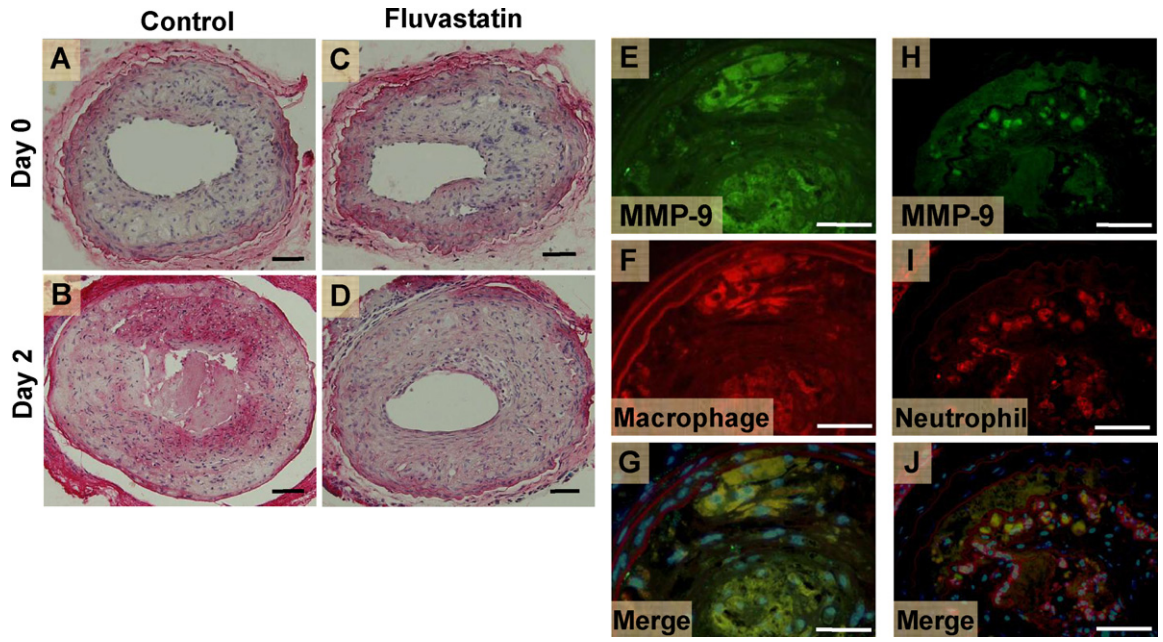

Fig. 5. Immunostaining of MMP-9 and identification of MMP-9-positive cells using immunofluorescent staining in the neointimal lesions of carotid arteries at days 0 and 2 after cuff placement. Immunoreactivity of MMP-9 at day 0 in the control (A) and fluvastatin (C) groups and at day 2 in the control (B) and fluvastatin (D) groups. Double fluorescence staining for MMP-9 was detected in the neointima and colocalized with markers of macrophages (F and G) and neutrophils (I and J) at day 2 in the control. Bars = 50 μm.

caused by rupture of the fragile and tortuous neovessels formed within the plaque [18–22].

The reduction of the plaque disruption rate by fluvastatin administration was associated with the preservation of collagen content as well as with a reduction in gelatinolytic activities in the vascular wall, which were decreased and enhanced, respectively, after cuff placement. It is assumed that proteinases, such as the MMP or cathepsin family, in the plaque are responsible for the plaque vulnerability, thereby favoring plaque rupture [23–27].

However, the exact proteinase involved in the destabilization of plaque remains uncertain. In the present study, we observed that fluvastatin decreased MMP-9 mRNA expression in the plaque between days 2 and 4 after cuff placement. In addition, the results of the time course of mRNA expression of major MMPs suggest that MMP-9 might play an important role in the process of plaque vulnerability, at least in this model. In fact, MMP-9 was deposited in the plaque lesions, and colocalized with macrophages and neutrophils. However, it is possible that other proteinases are also involved in plaque vulnerability. It should be noted that we observed a decrease in type I collagen mRNA expression in fluvastatin-treated disrupted regions compared with those in control mice; this is consistent with previous *in vitro* observations, which have demonstrated that statin, including fluvastatin, suppresses type I collagen mRNA in various cell types [28,29]. Nevertheless, much high contents of collagen were detected in the fluvastatin group, suggesting that proteolytic activity in the disruption-prone region was strongly attenuated by fluvastatin treatment.

TIMPs are a family of naturally occurring specific inhibitors of MMPs whose activity in atherosclerotic plaques seems to correlate with decreased MMP activity and hence reduced matrix remodeling [30,31]. In the present study, we observed that fluvastatin had no effect on TIMP-1 or -2 mRNA expression with the exception of TIMP-1 mRNA expression at day 2, which is consistent with the previous observation by Luan et al. that lovastatin has no effect on TIMP-1 or -2 secretion or on mRNA expression in rabbit smooth muscle cells (SMCs) [32]. The present results further confirm that fluvastatin reduced gelatinase activity in the intima through the reduction of MMP-9 expression.

The present results clearly show that neutrophil infiltration into the vascular wall after cuff placement was significantly attenuated by fluvastatin treatment. We also observed that fluvastatin suppressed ICAM-1 and VCAM-1 mRNA expression after cuff placement, although this difference did not reach statistical significance. In addition, fluvastatin significantly reduced the E-selectin and P-selectin mRNA levels in cuffed arteries. These results are consistent with those of previous reports demonstrating that statins inhibit interactions between leukocytes and endothelial cells through a reduction in adhesion molecule expression [33–36], and also decrease the number of inflammatory cells within atherosclerotic plaques and ischemic tissues [37,38].

Some phenotypic characteristics of plaques, such as collagen content, necrotic core size, the SMCs/macrophage ratio and fibrous cap thickness, have been widely used as indirect indicators of their stability [39]. Long-term administration of statins leads to this phenotype [11–13], resulting in plaque stability. In the study by Johnson et al., long-term treatment with pravastatin inhibits acute plaque rupture, which occurs at high frequency in the brachiocephalic arteries of male apoE-deficient mice after 8–40 weeks of fat feeding [14]. They showed that long-term treatment with pravastatin causes an increase in fibrous cap thickness and a decrease in plaque lipid content, suggesting that long-term statin treatment leads to changes in plaque phenotypes in apoE-deficient mice. However, in the present model, we found that the relatively short-term administration of statin may act directly on cells, contributing to the process of cuff-induced plaque disruption rather than statin-induced phenotypic changes in arterial walls. In fact, no phenotypic changes in plaques were observed after fluvastatin administration for 3 days just before cuff placement. The observed beneficial effects of short-term statin use are similar to the acute benefits associated with the early initiation of statin treatment in ACS [6,7].

In the present study, although we used the dose of fluvastatin for mice that was comparable with plasma fluvastatin concentration accomplished after oral administration of its clinical doses (10–20 mg) in humans [15], the implication of the present study may be restricted to this dose. Thus, further studies will be necessary to study several doses, which would confirm the physiological

and clinical relevance of this model. It must be noted that the present model does not completely reproduce human plaque rupture, the final event in a long and complex pathophysiological process. However, this model appears to be analogous to the events that occur to some extent in human plaque rupture: a reduction in collagen content, the presence of MMPs, and an increase in apoptotic cells and inflammatory cells in the plaque lesions before thrombosis formation, followed by neointima cracks and thrombotic occlusion of the artery at the site of the presumed rupture [10]. In the present study, these processes leading to plaque disruption seem to be inflammatory responses triggered by the cuff placement. Statin appears to prevent plaque disruption through the reduction of inflammatory responses, including leukocyte infiltration and MMP-9 expression, which are also observed in human plaque rupture regions. However, it is possible that other observed pleiotropic effects of statins, such as improved endothelial function [40] and reduced thrombogenic response [41,42], may be involved in the statin effect.Acknowledgments

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