ACAT inhibitor F-1394 prevents intimal hyperplasia induced by balloon injury in rabbits

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Abstract **Acyl-CoA:cholesterol acyltransferase (ACAT) is thought to contribute significantly to lipid deposition in macrophages, which subsequently leads to the initiation and progression of atherosclerosis. The aim of the present study was to examine the influence of hypercholesterolemia on arterial hyperplasia induced by endothelial denudation and the direct effect of ACAT inhibition on lesion formation. Rabbits were fed either a cholesterol diet or a regular diet for 4 weeks, and then the left common carotid arteries were denuded of endothelium. After the operation, all rabbits were kept on the regular diet for 2–6 weeks. Two weeks after the denudation, the degree of intimal thickening and the number of proliferating cells (which were immunohistologically identified to be smooth muscle cells) were similar in hypercholesterolemic and normolipidemic rabbits. After that, both parameters progressively increased in hypercholesterolemic rabbits but declined in normolipidemic rabbits. Macrophages were apparent in the lesions only in hypercholesterolemic rabbits. Next, the effect of the ACAT inhibitor, (1*S*,2*S*)-2-[3-(2,2-dimethylpropyl)-3-nonylureido] cyclohexane-1-yl 3-[(4*R*)-*N*-(2,2,5,5-tetramethyl-1,3-dioxane-4-carbonyl)amino]propionate (F-1394), on neointimal formation in hypercholesterolemic rabbits was examined. Oral administration of F-1394 significantly reduced neointimal thickening and the extent of macrophages in lesions without affecting serum cholesterol levels. These results suggest that hypercholesterolemia causes macrophage-derived foam cell accumulation in lesions, and that the progression of lesions is accelerated by the presence of macrophages. Moreover, the study shows that F-1394 prevents neointimal formation even in the presence of hypercholesterolemia, indicating that F-1394 may be useful for treating restenosis after percutaneous translumenal coronary angioplasty in hyperlipidemic patients.** —Aragane, K., K. Fujinami, K. Kojima, and J. Kusunoki. **ACAT inhibitor F-1394 prevents intimal hyperplasia induced by balloon injury in rabbits. *J. Lipid Res.* 2001.** 42: **480–488.**

Supplementary key words intimal thickening • acyl-CoA:cholesterol acyltransferase

Acute mechanical injury to the arterial vessel wall results in neointimal hyperplasia (1–4). Neointimal formation is due, at least in part, to the migration and proliferation of cellular components in the medial and intimal compartments of the arterial vessel (5–8). This sequence of events is believed to describe the restenosis that occurs after angioplastic treatment in humans (9–13). The restenotic lesion is predominantly composed of smooth muscle cells (SMC) and the extracellular matrix that they elaborate (14). At present, antithrombotic agents are the only clinically available drugs for treating restenosis after angioplasty, but these drugs do not inhibit neointimal hyperplasia.

It has been documented that macrophage-derived foam cells constitute a significant proportion of the cells in atherosclerotic lesions, both in humans and in most animal models (15–22). In addition, it is believed that acyl-CoA: cholesterol acyltransferase (ACAT) in the arterial wall is involved in the initiation and progression of atherosclerosis, and that the accumulation of esterified cholesterol via the ACAT pathway in macrophages and SMC is an essential step in the progression of atherosclerotic lesions (23).

While the main emphasis in the development of restenotic lesions has been placed on SMC hyperplasia, a role for macrophage-derived foam cells has been emphasized in premature atherosclerotic lesions. Although macrophage-derived foam cells are present in restenotic lesions after directional coronary atherectomy or percutaneous translumenal coronary angioplasty (PTCA) (24, 25), little is known about their role. In addition, a number of cytokines released from macrophages, such as platelet-derived growth factor (PDGF), basic fibroblast growth factor, heparin-binding epidermal growth factor-like growth factor (HB-EGF), monocyte chemotactic protein 1 (MCP-1), vascular endothelial growth factor, colony-stimulating factors, interleukin 1, interleukin 8, and others, may be involved in the development of atherosclerosis (26, 27). It is possible that the cytokine-releasing function of macrophage-derived foam cells may play a part in the development of

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; BrdU, bromodeoxyuridine; CHO(+), prefed with a cholesterol diet; CHO(−), prefed a normal diet; HB-EGF, heparin-binding epidermal growth factor-like growth factor; HDL, high density lipoprotein; MAb, monoclonal antibody; MCP-1, monocyte chemotactic protein 1; PDGF, platelet-derived growth factor; PTCA, percutaneous translumenal coronary angioplasty; SMC, smooth muscle cell; TC, total cholesterol.

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intimal hyperplasia after balloon angioplasty, as well as in the formation of atherosclerotic lesions.

In the present study, we sought to elucidate the role of macrophage-derived foam cells in lesion formation induced by balloon injury by examining the morphological changes that result from alterations in arterial ACAT activity. More precisely, we examined the influence of hypercholesterolemia on the arterial hyperplasia induced by endothelial denudation and the direct effect of a potent ACAT inhibitor, (1*S*,2*S*)-2-[3-(2,2-dimethylpropyl)-3-nonylureido] cyclohexane-1-yl 3-[(4*R*)-*N*-(2,2,5,5-tetramethyl-1,3-dioxane-4-carbonyl)amino]propionate (F-1394), on lesion formation. We have already reported that F-1394 is equally effective in inhibiting ACAT activity in intestine, liver, and macrophages (28, 29).

MATERIALS AND METHODS

Chemicals and antibodies

F-1394 (Fujirebio, Tokyo, Japan) was used to potently inhibit ACAT activity. A final F-1394 concentration of 100 mg/ml in a solution of 1 volume of ethanol, 1 volume of propylene glycol, and 7 volumes of Tween 80 was used for oral administration. The following monoclonal antibodies (MAb) were used for immunohistochemical analysis: HHF-35 (mouse MAb anti-human α-smooth muscle actin), 1A4 (mouse MAb anti-human α-smooth muscle actin), RAM-11 (mouse MAb anti-rabbit macrophage), and Bu20a [mouse MAb anti-bromodeoxyuridine (BrdU)]. All MAb were purchased from Dako Japan (Kyoto, Japan). All other reagents used were standard, commercial high-purity materials.

Animals and study protocols

Male New Zealand white rabbits (Kitayama Labes, Nagano, Japan) weighing 1.5–2.0 kg were maintained separately in a temperature- and humidity-regulated room (22 ± 2°C, 55 ± 15%) under controlled lighting (12-h light:dark cycle). They had free access to tap water and were fed 100 g of commercial regular chow (RC-4; Oriental Yeast, Tokyo, Japan) per day before the experiments were conducted.

Two separate in vivo experiments were undertaken. The first experiment evaluated the influence of hypercholesterolemic conditions on the formation of a lesion induced by balloon injury. For 4 weeks, the rabbits were fed, per day, 100 g of regular chow either with or without 0.5% cholesterol (Oriental Yeast). Denudation of the left common carotid artery was performed on each rabbit by passing a 3F balloon catheter (Fogarty catheter; Baxter Healthcare, Deerfield, IL) through the external branch under constant pressure. For all surgical procedures, rabbits were anesthetized at 1 ml/kg with a solution containing sodium pentobarbital (50 mg/ml) (Dainippon Pharmaceutical, Osaka, Japan) in saline. After ballooning, all rabbits were fed, per day, 100 g of the regular chow and were killed 2, 4, or 6 weeks after surgery. To examine cell proliferation in the lesions, BrdU, a thymidine analogue that is incorporated into the DNA of cells in S phase, was continuously infused through a miniaturized osmotic pump (Alza 2ML-1; Alzet Osmotic Pumps, Durect, Cupertino, CA) implanted in a cervical subcutaneous pocket 3 days before killing the rabbits.

The second experiment was performed to determine the contribution of ACAT activity to lesion formation in hypercholesterolemic rabbits. Rabbits fed the 0.5% cholesterol diet for 4 weeks underwent endothelial denudation of the left carotid arteries. On the day of surgery, animals were assigned to either one of two groups to make the mean serum total cholesterol (TC) levels in the two groups similar. Then, either F-1394 (100 $mg \cdot kg^{-1} \cdot day^{-1}$) or vehicle alone (1 $ml \cdot kg^{-1} \cdot day^{-1}$) was administered to the animals, which were then fed regular chow for 6 weeks. Surgical procedures were as described above.

Measurement of serum TC levels

Blood samples were obtained from the ear vein under fasting conditions. The concentrations of TC were measured enzymatically, using a commercial assay kit (Cholesterol E-HA Test Wako; Wako Pure Chemical Industries, Osaka, Japan). High density lipoprotein (HDL) cholesterol levels were measured with the same kit used for measuring TC concentrations, followed by fractionation with HDL cholesterol precipitation reagent (Wako Pure Chemical Industries). Concentrations of non-HDL cholesterol were calculated by subtracting HDL cholesterol levels from TC levels.

Histochemistry

Each animal was anesthetized with a sodium pentobarbital solution and exsanguinated via the right common carotid artery. Immediately afterward, the left common carotid artery was carefully removed and stripped of extraneous tissue. Vessels were then fixed in 20% buffered formalin (Mildform 20 N; Wako Pure Chemical Industries), divided into 5-mm segments, and embedded in paraffin wax. Serial cross sections were cut and used for Azan-Mallory and hematoxylin-eosin staining and for immunohistochemical staining using 1A4, RAM-11, and Bu20a MAb. Some sections were immunohistochemically stained with both HHF-35 and Bu20a. Antibody binding was visualized with the aid of a commercial kit (Vectastain Universal Elite ABC kit; Vector Laboratories, Burlingame, CA). Diaminobenzidine tetrahydrochloride or True Blue solution (KPL, Gaithersburg, MD) was used as a peroxidase substrate. All procedures were performed according to the protocol recommended by the manufacturers of the kit. For quantitative analysis, the image of each stained cross section was captured by a computer through a microscope (Nikon, Tokyo, Japan) attached to a 3-CCD color video camera (KY-F55MP; Victor Company of Japan, Yokohama, Japan), and the extent of the area of interest was calculated with the aid of image analysis software (Mac Scope; Mitani, Tokyo, Japan). Intimal and medial areas were measured in Azan-Mallory-stained slides. Proliferating cells were counted using BrdU-stained slides. Six segments from each carotid artery were used for quantitative and statistical analyses.

Statistical analysis

Data are presented as means ± standard error. The significance of differences between data collected from two groups was determined by Wilcoxon's rank sum test, Student's *t*-test, or the Aspin-Welch test (SAS System; SAS Institute; Cary, NC), with $P < 0.05$ considered significant.

RESULTS

Influence of hypercholesterolemic conditions on lesion formation

Table 1 shows serum TC levels and body weights of rabbits at the indicated time points after ballooning. All rabbits progressively gained weight, but starting only 2 weeks after surgery. No significant differences in body weights between the group prefed with a cholesterol diet [CHO(+)]

TABLE 1. Serum TC level and body weight in rabbits undergoing balloon injury

Weeks after Ballooning	Prefeeding	Serum TC	Body weight
		mg/dl	*kg*
Week 0	Normal	47 ± 3	2.33 ± 0.02
	Cholesterol	1,269 ± 96[a]	2.32 ± 0.02
Week 2	Normal	69 ± 12	2.25 ± 0.08
	Cholesterol	714 ± 87[a]	2.35 ± 0.07
Week 4	Normal	42 ± 4	2.51 ± 0.07
	Cholesterol	346 ± 86[b]	2.52 ± 0.06
Week 6	Normal	41 ± 8	2.67 ± 0.05
	Cholesterol	110 ± 62	2.63 ± 0.09

Rabbits were fed a 0.5% cholesterol diet or a normal diet for 4 weeks prior to endothelium denudation of the left carotid artery. After the operation, all animals were fed a normal diet. The data are expressed as means ± SE for 5–15 rabbits.

[a] $P < 0.001$ compared with rabbits fed a normal diet at the same time point.

[b] $P < 0.01$ compared with rabbits fed a normal diet at the same time point.

and the group prefed a normal diet [CHO(−)] were observed during the experimental period. Serum TC levels in the CHO(−) group remained fairly constant over time, at about 50 mg/dl. In contrast, TC levels in the CHO(+) group were 1,269 ± 96 mg/dl at week 0 and then declined rapidly. At week 6, TC levels in the CHO(+) group were 110 ± 62 mg/dl, not significantly different from TC levels in the CHO(−) group.

Results of the quantitative analysis of carotid artery cross sections are shown in **Table 2**. In the CHO(−) group, the intima/media ratio (which is a measure of the degree of neointimal thickening) was 0.08 ± 0.01, 2 weeks after the operation; this value was lower at later time points. Although in week 2 the intima/media ratio in the CHO(+) group was the same as that in the CHO(−) group, the ratio in the CHO(+) group increased with time. In week 6, the ratio in the CHO(+) group was approximately 6-fold greater than that in the CHO(−) group. There were significant intergroup differences in intima/media ratios, but not in TC levels. Similarly, in the CHO(−) group the number of BrdU-positive cells (which is a measure of cell proliferation) was 26 ± 6 cells per 5-mm cross section 2 weeks after the denudation; this number decreased progressively with time. In contrast, although in week 2 the number of proliferating cells in the CHO(+) group was almost the same as that in the CHO(−) group, the number of proliferating cells at later time points was significantly higher in the CHO(+) group. The value for macrophage occupancy in the CHO(+) group was 0.46 ± 0.18% of the 5-mm cross section at week 2; this value increased progressively with time and was approximately 3-fold greater at week 6 than at week 2. In contrast, macrophages were never detected in the CHO(−) group.

Figure 1 shows photographs of carotid artery cross sections stained with Azan-Mallory, antimacrophage MAb, anti-SMC MAb, or anti-BrdU MAb. These sections were obtained from CHO(−) and CHO(+) groups 6 weeks after balloon angioplasty. In the cross section from the CHO(−) animal the neointima is barely visible (having an intima/media ratio of 0.02), and SMC were the only cells in the lesion (Fig. 1A and E). Neither macrophages nor proliferating cells were found in sections from CHO(−) animals (Figs. 1C and G). In contrast, a huge lesion, having an intima/media ratio of 1.33 and consisting of SMC and macrophages, was observed in the cross section from the CHO(+) animal (Fig. 1B, D, and F). Macrophages appeared to have penetrated the medial layer of the artery and to have stuck to the internal elastic lamella in the neointima, while SMC filled the lesion and occupied the subendothelial space (Fig. 1B and D). In addition to macrophages, actively proliferating BrdU-positive cells were observed in the lesions in the CHO(+) animal; BrdU-positive cells appeared to be surrounding the macrophages (Fig. 1H). Moreover, according to double staining with anti-SMC and BrdU MAb, BrdU-positive cells were coincident with SMC but not with macrophages (see **Fig. 2**; arrows show double-stained cells).

Effect of ACAT inhibition on lesion formation

Serum TC levels, which increased by feeding the rabbits the 0.5% cholesterol diet for 4 weeks, reached approximately 1,250 mg/dl in week 0 (**Fig. 3**) and then decreased rapidly in the untreated (control) and F-1394-treated groups when the diet was changed to the regular chow. No significant differences in serum TC levels were observed between the untreated and F-1394-treated groups during the dosing period (week 0–6). No significant differences in serum HDL cholesterol and non-HDL cholesterol levels were observed between the two groups at week 6 (control group, 37 ± 5 and 108 ± 55 mg/dl, respectively; F-1394-treated group, 28 ± 3 and 19 ± 5 mg/dl, respectively).

TABLE 2. Intima/media ratio, macrophage occupancy, and cell proliferative activity in the carotid artery 2, 4, and 6 weeks postinjury

	2 Weeks			4 Weeks			6 Weeks		
	CHO(−)	CHO(+)	*P*	CHO(−)	CHO(+)	*P*	CHO(−)	CHO(+)	*P*
Intima/media ratio	0.08 ± 0.01	0.08 ± 0.01	0.9112	0.02 ± 0.01	0.08 ± 0.02	0.0051	0.03 ± 0.01	0.17 ± 0.06	0.0351
Macrophage (%)	0.00 ± 0.00	0.46 ± 0.18		0.00 ± 0.00	1.04 ± 0.52		0.00 ± 0.00	1.28 ± 0.72	
BrdU (cells)	26 ± 6	24 ± 4	0.8051	9 ± 3	49 ± 15	0.0156	3 ± 1	51 ± 20	0.0232

The data are expressed as means ± SE of three to five rabbits (six sections per rabbit). Carotid artery was serially cut into 5-mm lengths and cross sections were prepared. CHO(−) and CHO(+) represent normal chow-fed group and cholesterol prefed group, respectively. Statistical analysis of macrophage data is not shown because all sections in the CHO(−) group showed a complete absence of macrophage.

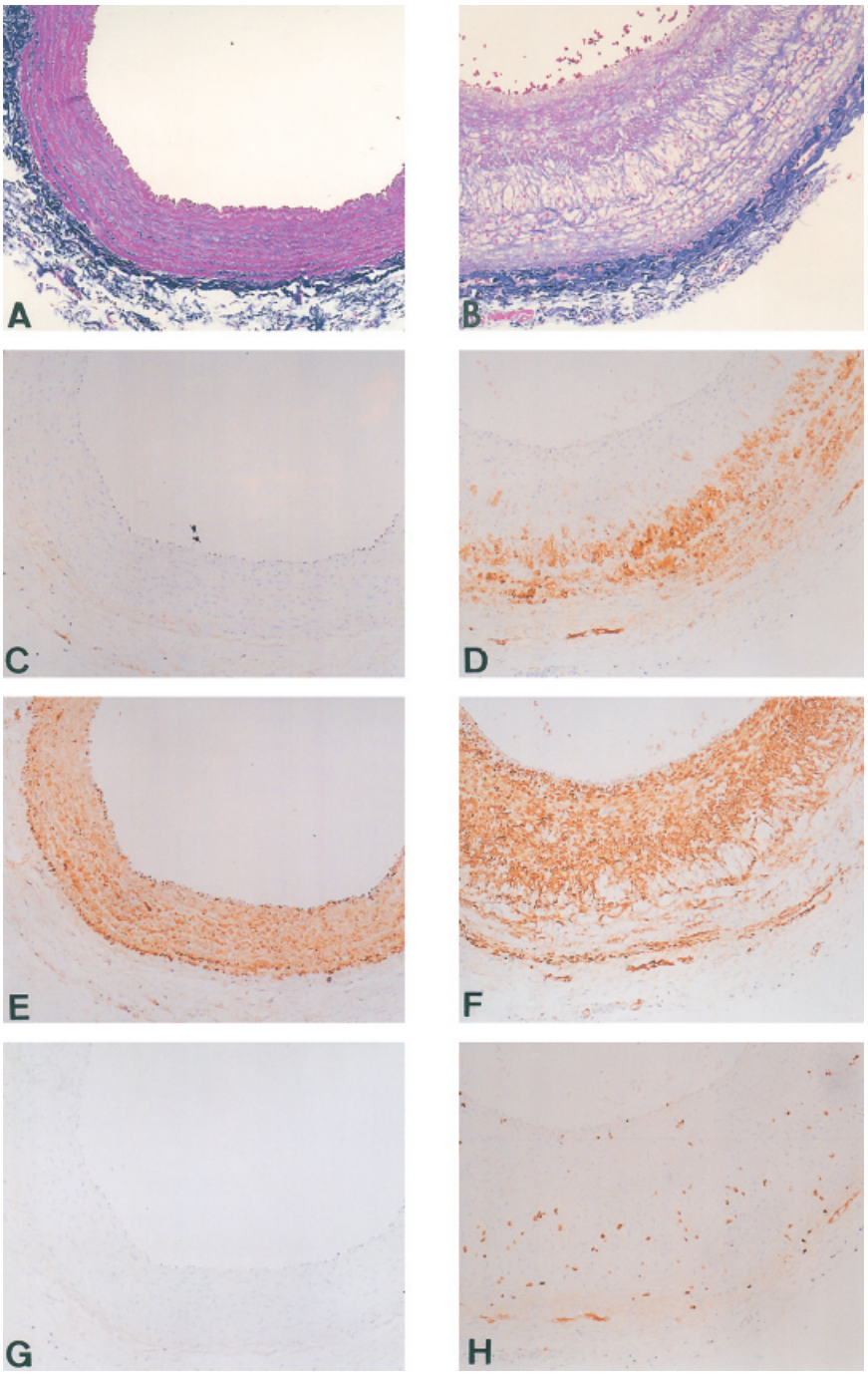

Fig. 1. Serial cross sections showing immunohistochemical staining of the left common carotid artery in rabbits prefed either a normal diet or a cholesterol diet. A and B: Azan-Mallory staining. C and D: Staining for macrophages. E and F: Staining for SMC. G and H: Staining for proliferating cells. A, C, E, and G: A representative animal in the normal diet group. B, D, F, and H: A representative animal in the cholesterol diet group. In (C–H), positive immunostaining is indicated by a brown color. Sections were taken 6 weeks after balloon angioplasty. Original magnification: ×25.

Figure 4 shows serial cross sections of common carotid arteries from untreated and F-1394-treated animals. Many vacuolar cells were found beside the internal elastic lamella in Azan-Mallory-stained sections from control animals (Fig. 4A, intima/media ratio of 0.415). Vacuolar cells were also stained with antimacrophage MAb (Fig. 4C); the main cellular components of the neointima were SMC (Fig. 4E). In contrast, smaller lesions were observed in cross sections from the F-1394-treated animals (Fig. 4B, intima/media ratio of 0.154). The neointima consisted almost entirely of SMC, and macrophages were rarely identified. As is shown in **Fig. 5**, in the sections of the left common carotid artery from control animals the intima/media ratio was 0.37 ± 0.5 (left). This ratio was significantly (64%) smaller in sections from F-1394-treated animals. Macrophages occupied approximately 1% of the lesion in the sections from control animals (right). The RAM-11-positive area was significantly (84%) smaller in F-1394-treated animals.

DISCUSSION

The present study was performed to determine *i*) the influence of hypercholesterolemic conditions on neointimal formation induced by endothelial denudation and

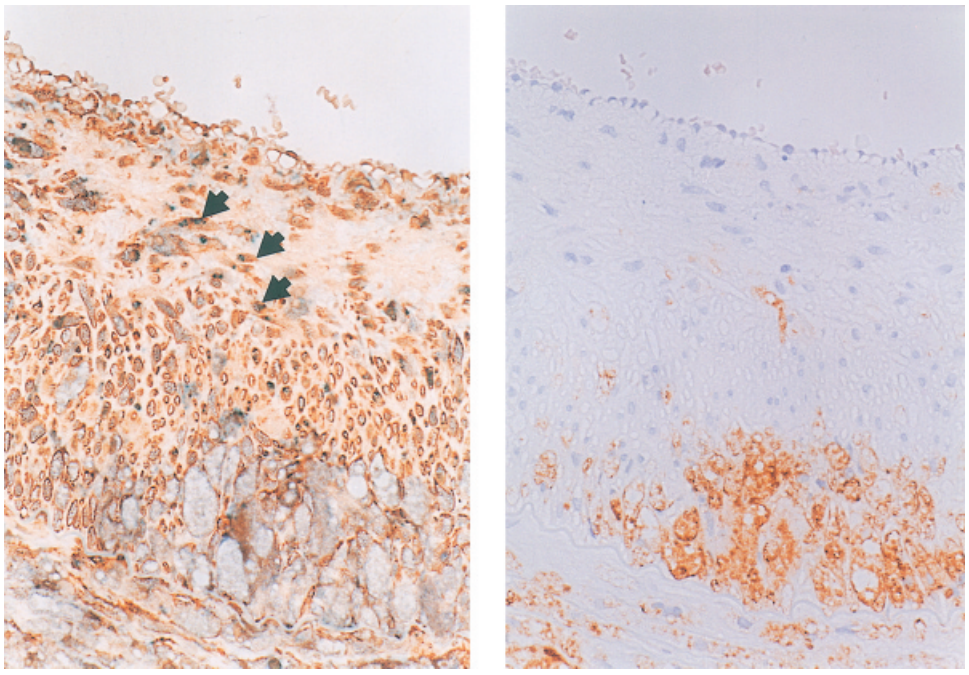

Fig. 2. Serial cross sections showing double immunohistochemical staining of the left common carotid artery in rabbits prefed a cholesterol diet. Left: Double staining of SMC and proliferating cells. Dark blue staining represents proliferating cells; brown represents SMC. Arrows indicate double-stained cells. Right: Immunohistochemical staining of macrophages only, indicated by brown color. Sections were taken 6 weeks after balloon angioplasty. Original magnification: ×100.

ii) the direct effect of an ACAT inhibitor on lesion formation. In this study, we used an orally active ACAT inhibitor, F-1394, to inhibit ACAT activity in vivo. It is well known that ACAT, which catalyzes cellular cholesterol esterification, is responsible for cholesterol absorption via the gut as well as for foam cell formation in arteries (30, 31). Thus, in addition to having an antiatherosclerotic effect, ACAT inhibitors are expected also to act as hypocholesterolemic agents by preventing cholesterol absorption. In fact, it has been well documented that ACAT inhibitors, including F-1394, decrease the serum TC levels in cholesterol-fed hypercholesterolemic animal models (32–37). We therefore had to establish a new protocol that would enable us to quantify the direct effect of F-1394 on neointimal formation, but exclude any secondary effects of its hypocholesterolemic action. The protocol consisted of inducing hypercholesterolemia in rabbits by feeding them a 0.5% cholesterol diet prior to endothelial denudation and F-1394 administration. The diet was then changed to regular chow to avoid the influence of F-1394 on cholesterol absorption. Moreover, animals were assigned to either the F-1394-treated or untreated group in a way that would ensure that each group's mean TC levels would be about equal before starting F-1394 treatment.

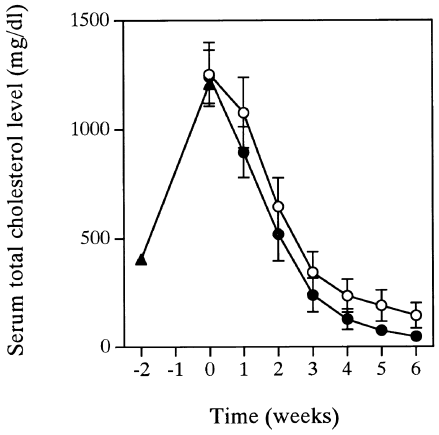

Fig. 3. Changes in serum TC levels. Rabbits were fed a 0.5% cholesterol diet for 4 weeks until week 0. The diet was then changed to regular chow. Rabbits were orally given F-1394 (closed circles) or its vehicle (open circles) from week 0 to week 6. Filled triangles show data obtained before the rabbits were divided into the two groups. Data are shown as means ± SE.

Before studying the effect of ACAT inhibition, we examined whether neointimal thickening induced by denudation of the carotid artery was more extensive under hypercholesterolemic conditions. Although serum TC levels in the CHO(+) group declined rapidly after the diet was changed to the regular chow, the rabbits were still hypercholesterolemic until at least 4 weeks after denudation, as is indicated in Table 1. In the normocholesterolemic rabbits, a neointimal lesion, having SMC as the sole cellular components, formed within 2 weeks after denudation and then began to heal (Table 2). This phenomenon is consistent with the so-called response-to-injury hypothesis (27), according to which stimuli of SMC growth, such as PDGF, act transiently on the arterial wall. On the other hand, under hypercholesterolemic conditions, the neointimal

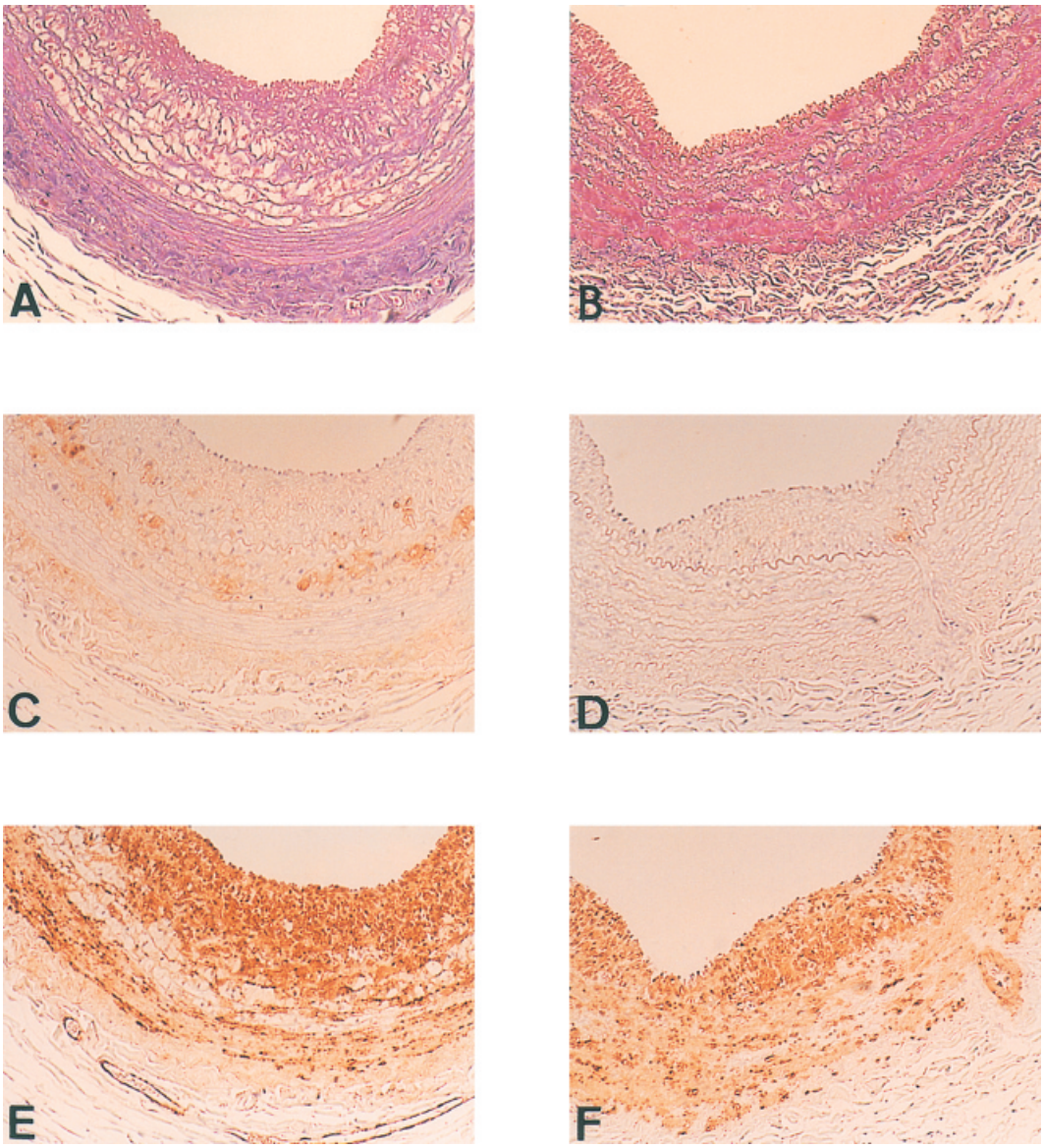

Fig. 4. Serial cross sections showing intimal lesion formation in the left common carotid artery of rabbits prefed the cholesterol diet. A and B: Azan-Mallory staining. C and D: Staining for macrophages. E and F: Staining for SMC. A, C, and E: A representative animal from the control group. B, D, and F: A representative animal from the ACAT inhibitor-treated group. In (C–F), positive immunostaining is indicated by a brown color. Original magnification: ×25.

hyperplasia induced by endothelial denudation advanced progressively (Table 2). Immunohistochemical analysis showed that in the CHO(+) group, the lesion not only contained SMC but also macrophages (Fig. 1). Moreover, 6 weeks after denudations, the SMC surrounding the macrophages were actively proliferating in the lesions of the CHO(+) animals (Fig. 2), even though TC levels were not different from those in normocholesterolemic rabbits (Table 1). These results indicate that neointimal formation is primarily associated with SMC proliferation at the lesion site. Furthermore, the presence of macrophage-derived foam cells at the lesion site may continuously stimulate SMC proliferation and, consequently, advance lesion formation. In other words, the progression of neointimal hyperplasia is unlikely to result from the high TC levels, but rather from foam cells that appear because of the induction of hypercholesterolemia in this model system. Thus, these findings suggest that macrophage-derived foam cells play a significant role in the progression of intimal thickening after balloon angioplasty.

SMC proliferation in the arterial wall is a major event in the development of atherosclerosis, including the development of restenosis after balloon angioplasty (38–42). The lesion induced by balloon angioplasty is exposed to many factors that stimulate SMC migration and proliferation. In particular, various factors released by macrophages have been reported to stimulate SMC hyperplasia at the lesion site (26, 27, 43, 44). However, the actual factor(s)

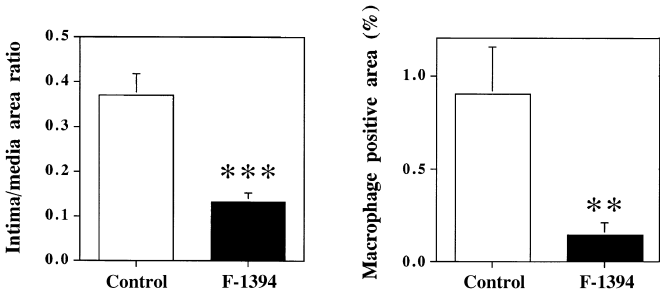

Fig. 5. Effect of ACAT inhibition on intima/media area ratio and macrophage-occupied area in common carotid arteries of rabbits prefed the cholesterol diet. Left: Intima/media ratio 6 weeks after balloon angioplasty. Data are presented as means ± SE for eight or nine animals (six sections per rabbit). Carotid arteries were serially cut by 5-mm lengths and cross sections were prepared. *** $P < 0.001$ versus control. Right: Macrophage-occupied area 6 weeks after balloon angioplasty. Data are presented as means ± SE for eight or nine animals (six sections per rabbit). Carotid arteries were serially cut by 5-mm lengths and cross sections were prepared. ** $P < 0.01$ versus control.

responsible for the accumulation of intimal SMC have not been determined. Ferns et al. (45) showed that a polyclonal antibody to PDGF inhibits the development of an intimal lesion in the carotid artery after balloon catheter de-endothelialization. On the basis of this finding, PDGF is a strong candidate for the major factor involved in restenosis after balloon angioplasty; the neointimal hyperplasia observed in the present study might be due to a continuous secretion of PDGF from macrophage-derived foam cells at the lesion site. On the other hand, it is well known that macrophages respond to cholesterol loading by altering their cellular metabolism. The chemokine MCP-1, which is responsible for monocyte migration, has been identified in macrophage-derived foam cells in atherosclerotic lesions (43, 46). Moreover, enhanced expression of MCP-1 mRNA was observed in macrophage-derived foam cells. Transplantation of bone marrow from mice overexpressing MCP-1 into irradiated apolipoprotein E-deficient mice increases the progression of atherosclerosis by increasing both macrophage numbers and oxidized lipid accumulation (47). In addition, apolipoprotein E-deficient mice lacking the receptor for MCP-1, CC chemokine receptor 2, have reduced atherosclerotic lesions as a result of reduced foam cell deposition and fewer fatty streaks (48). Consequently, it is now believed that MCP-1 has a potential role in the recruitment of monocyte-macrophages into the arterial wall and, thereby, in the subsequent progression of the atheroma. In the present study, the accumulation of macrophages in the lesion may have been induced by MCP-1 secreted by macrophage-derived foam cells that appeared as a result of the hypercholesterolemic conditions. These macrophages may have been the cause of the advanced neointimal formation seen in CHO(+) animals, because macrophages also produce potent mitogenic factors for SMC, such as PDGF and HB-EGF (27). On the basis of these findings, it can be inferred that macrophage-derived foam cells make an important contribution to restenosis in the neointima in the coronary artery after PTCA.

Consistent with this conclusion, F-1394 reduced both neointimal formation and the macrophage-occupied area in atherosclerotic lesions in rabbits prefed a cholesterol diet, without affecting serum TC levels (Figs. 3–5). Previous studies by our group have shown that F-1394 does not affect the proliferation of cells, such as SMC (K. Aragane, K. Fujinami, K. Kojima, and J. Kusunoki, unpublished data), and the median inhibitory concentration of F-1394 in relation to macrophage ACAT activity is 0.032 μM (28). When F-1394 was administered at 100 mg/kg to normal rabbits for 7 days, the maximum concentration (C_{max}) and C_{24} for the unchanged form of F-1394 were 0.41 and 0.010 μM, respectively, on day 7 (as determined by liquid chromatography-tandem mass spectrometry; K. Aragane, K. Fujinami, K. Kojima, and J. Kusunoki, unpublished data). Furthermore, we have demonstrated that the concentration of the unchanged form of F-1394 was sufficient to inhibit ACAT activity at the arterial wall (K. Aragane, K. Fujinami, K. Kojima, and J. Kusunoki, unpublished data). Therefore, the reduction in neointimal formation in F-1394-treated rabbits in this study must have occurred as a result of the inhibition of arterial ACAT activity. In turn, this suggests that macrophage-derived foam cells decrease as a result of direct inhibition of ACAT, leading to an attenuation of neointimal formation. Bocan et al. (49) reported the seminal finding that CI-976, an ACAT inhibitor, reduces the size of atherosclerotic lesions without significantly reducing serum TC levels in rabbits fed a cholesterol diet. However, their protocol was more susceptible to a hypolipidemic effect of the ACAT inhibitor. Because the protocol used in our study is not susceptible to the hypolipidemic effect of F-1394 administration, we can conclude that ACAT inhibition has a direct effect on atherosclerosis.

In mammals, two different ACAT genes, ACAT-1 and ACAT-2, have been identified (50, 51). The results of immunodepletion studies suggest that the ACAT-1 protein is responsible for most ACAT activity present in homogenates of the various adult tissues and cells examined so far; in humans these include liver, hepatocytes, adrenal gland, macrophages, and kidney, but not intestines (52). In addition, using a specific ACAT-2 antibody, it has been shown that ACAT-2 performs a significant catalytic role in fetal and intestinal enterocytes in humans (53). Two research groups have reported that a complete lack of ACAT-1 function in genetically hyperlipidemic mice results in massive dermal xanthomatosis, dull fur, pruritus, hair loss, excoriations, and grossly thickened skin from accumulated crystals of free cholesterol (54, 55). In the present study, severe problems due to ACAT inhibition were not observed. Furthermore, a long-term administration of F-1394 did not induce such problems in apolipoprotein E-deficient mice fed a Western-type diet or in Watanabe heritable hyperlipidemic rabbits (K. Aragane, K. Fujinami, K. Kojima, and J. Kusunoki, unpublished data). These results raise the important question of whether a therapeutic strategy for atherosclerosis using ACAT inhibi-

tion would be beneficial in a setting of severe and persistent hyperlipidemia. Although there is no clear explanation for the discrepancy between genetic and pharmacological inhibition of ACAT activity, it might be due to the fact that F-1394 does not completely inhibit enzyme activity in vivo at all times because the degree of the inhibitory effect and its duration absolutely depend on the pharmacokinetic features of the compound.

In summary, this study suggests that the macrophage-derived foam cells that appear as a result of hypercholesterolemic conditions promote SMC proliferation and neointimal formation in arteries, after endothelial injury inflicted by balloon catheter. ACAT inhibition by F-1394 prevents or reduces intimal thickening by decreasing the accumulation and/or recruitment of macrophages at the lesion site without affecting serum TC levels. On the basis of these findings, macrophage-derived foam cells could represent an important target in the treatment of atherosclerotic disease and F-1394 may be used as a potential treatment for atherosclerosis, including PTCA-induced restenosis.

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