Fluvastatin Reduces Tissue Factor Expression and Macrophage Accumulation in Carotid Lesions of Cholesterol-Fed Rabbits in the Absence of Lipid Lowering

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Abstract—The expression of tissue factor (TF), mainly by infiltrated inflammatory cells, has been shown to be responsible for the thrombogenicity associated with atheroma. The contribution of the nonlipid-related effects of statins to the clinical benefits of statin therapy is currently under intense investigation. In this study, we evaluated the ability of fluvastatin to modulate TF expression and macrophage accumulation in rabbit carotid intimal lesions independently of cholesterol lowering. Male rabbits were fed for 30 days a 1% cholesterol-rich diet with or without fluvastatin at 5 mg/kg per day. Two weeks from the start of treatment, a silastic collar was placed around the carotid artery. Fifteen days later, the animals were killed, and carotid segments were excised and processed. The atherogenic diet caused a consistent increase in plasma cholesterol levels (610 ± 231 mg/dL versus 50 ± 9 mg/dL at baseline), which were not affected by fluvastatin (603 ± 248 mg/dL). In the rabbits fed a high cholesterol diet without fluvastatin, an intimal lesion with macrophage accumulation and TF expression was detected. Fluvastatin significantly reduced TF and macrophage content of the lesion (−50% for both). Results indicate that fluvastatin may attenuate the inflammatory and thrombogenic potential of atherosclerotic lesions through a mechanism(s) other than cholesterol reduction, providing new insight regarding the complex mode of action of statins. (Arterioscler Thromb Vasc Biol. 2002;22:692-698.)

Key Words: tissue factor ■ thrombosis ■ inflammation ■ statins ■ collar model

Tissue factor (TF) is a transmembrane glycoprotein that binds factor VII/VIIa, triggering the downstream coagulation pathway that leads to fibrin deposition.1 Immunohistochemical studies have identified a selective distribution of TF in normal tissues.2 In the vessel wall, TF is abundant in the adventitia, is variably expressed in the media, and is virtually absent in the intima.3,4 However, chemical or mechanical injury of the vessel wall of experimental animals has been shown to induce a consistent expression of TF in the intima and in the media.5,6 Moreover, in human atherosclerotic endarterectomy specimens, TF mRNA and antigen are detectable in mesenchymal-like intimal cells (presumably vascular smooth muscle cells), in the extracellular matrix, and mainly in foam cells and monocytes adjacent to cholesterol clefts.7−9 This suggests that TF participates in the development of atherosclerotic lesions and mediates their thrombogenicity as well.10 Statin therapy is known to reduce the incidence of cardiovascular events and death, probably by functional changes of atherosclerotic lesions.11−14 These benefits are mainly ascribed to the strong lipid-lowering properties of this class of drugs.11−14 However, increasing evidence suggests that many of the beneficial effects exerted by statins on vascular cells may be independent of cholesterol lowering.15−19 In particular, statins have been shown to reduce TF expression in cultured macrophages through a nonlipid-related effect involving the reduced formation of a geranylgeranylated protein(s) required for the proper synthesis of TF.20 Aikawa et al21 have recently shown that cholesterol lowering by cerivastatin is associated with reduced TF expression in macrophages infiltrating aortic lesions of Watanabe heritable hyperlipidemic rabbits. However, the contribution of a cholesterol-independent action of the statin to this antithrombotic effect could not be ruled out and would appear to be relevant as a mechanism underlying those clinical benefits of statin therapy that cannot fully be explained by lipid lowering.22

The positioning of a hollow silastic collar around the carotid artery of rabbits has been previously shown to rapidly cause atherogenic events, such as leukocyte infiltration, smooth muscle cell proliferation, and extracellular matrix deposition, in the presence of an intact endothelium.23−26 Previous studies performed in this animal model have shown that statins reduce the extent of collar-induced carotid lesions,
either in normocholesterolemic or in hypercholesterolemic rabbits (M.R. Soma, unpublished data, 1996), without altering plasma cholesterol levels. Thus, the collar approach appears to be suitable for evaluating the potential nonlipid-related effects of statins in in vivo models of experimental atherosclerosis. On this basis, the present study was designed to investigate the ability of a relatively short-term treatment with fluvastatin to modulate TF expression and macrophage accumulation in collar-induced rabbit carotid lesions independently of cholesterol lowering.

Methods

Study Design

In preliminary experiments, we assessed TF expression and macrophage accumulation in collar-induced carotid intimal lesions of normocholesterolemic rabbits (n=6). No TF expression or macrophage infiltration was observed under this experimental condition. Because cholesterol feeding has been previously shown to increase intimal TF expression, experiments were performed in cholesterol-fed rabbits, as described below.

Twenty-eight New Zealand White male rabbits (2.3 to 2.6 kg body weight) were fed a normal diet plus a cholesterol-rich diet, giving a final dietary dose of 1% cholesterol for 4 weeks (HC rabbits). Half of the animals simultaneously received fluvastatin (Novartis) at 5 mg/kg per day (HC+F rabbits). The daily amounts of cholesterol and fluvastatin were given in the morning; each was mixed in 20 g of food pellets. Normal chow up to 150 g was provided after all the medicated diet had been eaten. Two weeks after the initiation of the cholesterol-rich diet, a carotid lesion was induced by perivascular injury in all animals (see below). After 4 weeks of treatment, the animals were euthanized with a lethal intravenous dose of urethane (10 mL, 25% aqueous solution), and vascular tissue was isolated and processed for histology. All experiments were performed in accordance with the guidelines for Animal Care and Treatment of the European Community.

Serum total cholesterol (TC) concentration was determined by enzymatic assay (Boehringer-Mannheim) 2 weeks after the initiation of the study and at its conclusion.

Perivascular Collar Insertion in the Carotid Artery

Carotid lesions were induced as previously described. Briefly, a nonocclusive biologically inert soft, hollow, silastic collar (Silicollar, MediGene Oy) was placed around both carotid arteries in anesthetized animals. In the sham-operated arteries, usually the left carotid arteries, the collar was removed just before the carotids were replaced anatomically and the wounds were sutured. Two weeks after carotid arterial injury, the animals were killed, and segments from the carotid arteries were excised as previously described. Microscopic analysis of histological sections from collared carotids always showed an intact morphology of the artery, with no disruption of either the internal elastic lamina (IEL) or external elastic lamina (EEL) and no laceration or necrosis of the tunica media.

Histology

The 1-cm-length central portion was formalin-fixed and embedded in paraffin. At least 3 series of 100 cross sections (5 μm) each were cut per rabbit. Each series was spaced 100 μm apart. From each series, randomly selected serial sections were stained with hematoxylin and eosin or were alternatively used for immunohistochemical detection of TF, rabbit macrophages, α-actin, or CD31 at 5 sections per parameter. Measurements were performed on 1 representative microphotograph for each series of sections. Luminal area, area surrounded by the IEL, and area surrounded by the EEL were measured by computer-assisted image analysis (OPTIMAS 6.2, Media Cybernetics). The following parameters were then determined: (1) intimal area=IEL−luminal area, (2) medial area=EEL−IEL, and (3) intima-to-media area ratio (I/M).

Figure 1. Immunohistochemical detection of TF in carotid intimal lesions of HC and HC+F rabbits. Collared carotids of HC rabbits show intense staining for TF (a and b). In carotid lesions of HC+F rabbits, TF immunostaining is also detectable (d and e) but much less so than in HC rabbits. No TF staining was present in sham-operated arteries from either HC or HC+F rabbits (c and f), except in the adventitia, where TF is constitutively expressed and not modified by drug treatment. All vascular cells within the lesion (macrophages, smooth muscle cells, and, focally, endothelial cells) exhibited immunopositivity for TF. Arrowheads indicate the IEL.

Figure 2. Quantitative analysis of TF expression within carotid intimal lesions of HC and HC+F rabbits. Data are reported as mean±SD of TF-positive percent area (A) and absolute TF-positive area (B) within the intima, measured by computer-assisted color image analysis. *P<0.05 vs HC rabbits.
Histochemical Assays
Immunohistochemistry was performed according to the ABC method (Vector). Antibodies used in the present study included mouse monoclonal antibodies against rabbit tissue factor (AP-1), rabbit macrophages (RAM11, DAKO), human α-smooth muscle actin (Sigma Chemical Co), human CD31 (platelet and endothelial cell adhesion molecule-1, DAKO), and proliferating cell nuclear antigen (PCNA, DAKO). For a negative control, primary antibody was omitted, and incubation was performed in the presence of normal horse serum (Vector Laboratories Inc). Immunopositivity to cell-specific antibodies was examined on sections adjacent to those used in the determination of TF-positive areas. The extent of intimal areas immunopositive to TF (TF area) and to RAM11 (MΦ area) were measured by computer-assisted image analysis (OPTIMAS 6.2). A threshold for immunostaining was defined by sampling, and morphometric data were collected in a blinded fashion.

For identification of proliferating macrophages, immunostaining with RAM11 was combined with PCNA labeling. Staining with PCNA antibody (1:50 dilution) was performed up to the diamino benzidine (DAB, Sigma) labeling stage. Sections were then blocked with 10% normal horse serum (Vector Laboratories Inc) and incubated overnight at 4°C with RAM11 primary antibody at 1:20 dilution. A biotinylated horse anti-mouse IgG secondary antibody (1:200 dilution, Vector Laboratories Inc) was added for 30 minutes and detected by ExtrAvidin-FITC conjugate (1:100 dilution, Sigma). Appropriate negative controls (without primary and/or secondary antibodies) were included. To identify intimal PCNA-positive cells colocalized with RAM11, each ×40 field was photographed first under an Axioskope (Zeiss) fluorescence microscope equipped with an FITC filter to obtain green fluorescence images of RAM11 staining and, subsequently, under white light to obtain color images of PCNA labeling. Immunocolocalization of the 2 antigens was then verified by computer-assisted subtraction of each fluorescence image to the corresponding color image. As a result of processing, cells positive to both PCNA and RAM11 (PCNA-(+) MΦ) appeared with black cytoplasmic staining and brown nuclear staining. The extent of PCNA-(+) MΦ was quantified by direct counting in a blinded fashion by 2 independent observers and was represented as the number of PCNA-(+) MΦ per section or as [PCNA-(+) MΦ/μMΦ area]×100, where MΦ area corresponds to the amount of intimal area immunopositive to RAM11 (measured in micrometers squared).

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was performed with an apoptosis detection kit (Boehringer-Mannheim), according to the manufacturer’s instructions. Staining in the absence of terminal deoxynucleotidyl transferase enzyme was performed as a negative control. Intimal TUNEL-positive cells were quantified with a ×40 objective and represented as the number of TUNEL-(+) cells per section or as [TUNEL-(+) cells/intimal area]×100, where the intimal area corresponds to the total extent of intimal lesion measured in micrometers squared.

Statistical Analysis
Data are expressed as mean±SD. Difference between groups was evaluated by 1-way ANOVA, followed by an unpaired Dunnett test.

Results
Plasma Cholesterol Levels
Mean serum TC levels were 50±9 mg/dL at the beginning of the study (before cholesterol feeding, n=28). After randomization of the animals into their respective treatments, TC levels in the control (HC) group rose to 578±179 mg/dL after the first 2 weeks on the atherogenic diet and remained increased at 4 weeks (610±231 mg/dL). Fluvastatin added to the cholesterol-rich diet did not influence the increase in plasma cholesterol (461±291 and 603±248 mg/dL after 2 and 4 weeks, respectively; P≥0.05 versus HC group).

TF Expression in Collar-Induced Intimal Hyperplasia of the Common Carotid Artery
Carotid lesions from HC rabbits showed intense TF immunostaining (Figure 1a and 1b). Sham-operated uncollared carotids did not develop intimal hyperplasia and displayed immunonegativity for TF only in the adventitia, where TF is known to be constitutively expressed (Figure 1c and 1f). In carotid intimal lesions of HC+F rabbits, TF immunostaining was significantly less than in lesions from HC animals (Figure 1d and 1e). Quantitative image analysis demonstrated a significant lower amount of lesion area occupied by TF, measured as absolute positive area (percent change versus control −60%, P<0.001) and
percentage of immunopositive area within the intima (percent change versus control = -50%, \( P<0.001 \); Figure 2).

### Macrophage Content in Collar-Induced Intimal Hyperplasia of the Common Carotid Artery

Carotid intimal lesions of HC rabbits were characterized essentially by conspicuous macrophage infiltration, identified by immunostaining for RAM11 (Figure 3a), whereas less macrophage infiltration was observed in fluvastatin-treated rabbits (Figure 3d). Smooth muscle cells, as noted by their \( \alpha \)-actin-positive staining (Figure 3b and 3e), could also be detected in lesions from both groups of animals. The luminal surface was lined with an intact endothelium, as assessed by CD31 staining (Figure 3c and 3f). The percentage area covered by macrophages, quantified by computer-assisted color image analysis, in HC and HC+F rabbits is shown in the Table. Linear regression analysis performed to test the association between TF expression and macrophage accumulation within the intima revealed a positive correlation between the absolute immunopositive areas for TF and RAM11 (\( R^2=0.5974, P<0.00001 \)).

### Macrophage Proliferation and Apoptotic Cell Death in Collar-Induced Intimal Hyperplasia of the Common Carotid Artery

To address the potential mechanisms responsible for the reduced macrophage number observed in carotid lesions from HC+F rabbits, we evaluated whether fluvastatin treatment was associated with inhibition of macrophage proliferation or the induction of apoptotic cell death. Double immunostaining of macrophages with RAM11 and PCNA indicated that the amount of intimal PCNA-positive cells that colocalized with RAM11 was not different between HC and HC+F rabbits (Figure 4). Total PCNA staining was also not affected by fluvastatin (data not shown). Similarly, fluvastatin did not influence the rate of apoptotic cell death, as assessed by TUNEL staining, which was found to be very low in both the experimental groups (Figure 5).

### Intimal Lesion Extent

The average cross-sectional area of the collar-induced carotid intimal lesions and the I/M area ratio in HC rabbits were 0.12±0.03 mm\(^2\) and 0.25±0.07, respectively. A reduction of
intimal hyperplasia was observed in HC+F rabbits, although it was not statistically significant (0.09±0.05 mm² for cross-sectional area of the collar-induced carotid intimal lesions and 0.19±0.11 for I/M ratio, P≥0.05 versus HC for both parameters).

Discussion
The present study shows that cells composing the intimal lesion of cholesterol-fed animals subjected to periarterial collaring are elicited to express TF, which confers a prothrombotic phenotype to the carotid artery. The concomitant administration of fluvastatin, a second-generation lipophilic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, markedly suppressed TF expression without affecting plasma cholesterol levels.

Aikawa and colleagues21,27 have recently reported that the enhanced expression of TF in atherosclerotic lesions of hypercholesterolemic rabbits is reversed by lowering the plasma cholesterol levels, either by dietary means or by cerivastatin treatment. These findings provided new insights regarding the potential mechanisms by which lipid lowering reduces the clinical complications of atherosclerosis. However, the contribution of cholesterol-independent actions of statins to the inhibition of TF expression in vivo remained to be established. In the present study, we showed that fluvastatin may exert direct nonlipid-related antithrombotic effects at the level of the arterial intima by reducing TF levels in experimental atherosclerotic lesions of cholesterol-fed rabbits without concomitant changes in serum cholesterol levels.

The absence of a cholesterol-lowering activity by fluvastatin is an important point of the present study, because it allowed us to assess the independence of the observed vascular effects from the hypocholesterolemic properties of statins. In general, HMG-CoA reductase inhibitors have been reported either to reduce29–32 or to leave unaltered33–35 the plasma cholesterol levels in hypercholesterolemic rabbits. Various reasons may presumably account for this seeming discrepancy. First, the effect of statins on the lipid profiles of hypercholesterolemic rabbits may differ in relation to the model of hypercholesterolemia (exogenous, ie, diet-induced, or endogenous, ie, genetically determined). Second, the effect may vary according to the study design (depending on the

Figure 5. Detection of apoptosis by TUNEL staining in carotid intimal lesions of HC and HC+F rabbits. A, Very low numbers of cells were stained positively by TUNEL method (green fluorescence) in carotid intimal lesions from HC rabbits (top left) and HC+F rabbits (bottom left). Right panels show TUNEL staining at higher magnification (boxed field). Arrowheads indicate the IEL; arrows, the endothelial layer. B, Quantitative analysis of TUNEL staining. Data are reported as number of TUNEL- (+) cells per section or as [TUNEL- (+) cells/intimal area]×100. Bars represent SD.
dose of drug and/or the levels of dietary cholesterol intake and depending on the duration of dietary treatment and/or exposure to the drug). In particular, it is worth mentioning that a cholesterol-rich diet such as that used in the present study has been reported to rapidly reduce liver HMG-CoA reductase activity in New Zealand rabbits by 90% of normal,16 a degree that may or may not be overcome by statin treatment. This latter observation may potentially explain the lack of hypcholesterolemic effect observed in the present study as well as in other experiments using statins in cholesterol-fed rabbits.33–35

The ability of fluvastatin to modulate vascular TF expression independent of cholesterol lowering is in agreement with numerous in vitro and in vivo observations, indicating that statins may exert several nonlipid pleiotropic effects. These effects include, for example, improvement of endothelial dysfunction, inhibition of smooth muscle cell migration and proliferation, reduction of matrix metalloproteinase secretion, and an increase of fibrinolytic activity.15–18 Moreover, statins have been recently shown to exert anti-inflammatory and immunosuppressive effects unrelated to lipid lowering.37–40

In general, the nonlipid-related effects of statins can be reversed by the addition of mevalonic acid or nonsterol mevalonate derivatives, directly implicating interference with the mevalonate pathway by statins. In addition, Weitz-Schmidt et al41 recently showed that statins, independent of their inhibition of HMG-CoA reductase activity, are able to directly block the interaction between leukocyte function–associated antigen type 1 (LFA-1) and intercellular adhesion molecule-1 by binding to a specific regulatory site on LFA-1. This latter mechanism, although investigated principally in vitro, provides important new insight to the growing evidence supporting the notion that statins possess significant lipid-independent properties.

The diminished TF expression reported in the present study reflects a reduction of monocye-derived macrophage content within the lesion. It has been previously shown that relatively long-term (32-week) treatment with cerivastatin suppresses macrophage proliferation in atheromas of Watanabe heritable hyperlipidemic rabbits fed a normal diet42; instead, we found no effect of fluvastatin on macrophage and total cell proliferation. Furthermore, no induction of apoptotic cell death by statin treatment could be detected. Thus, it is likely that in the present study, fluvastatin influenced monocyte recruitment in the carotid lesion, an effect that may have several molecular bases. First, statins decrease the expression of monocyte chemoattractant protein–1,42 which participates in monocyte recruitment into the vascular wall. Second, statins downregulate the expression of cell surface integrins, such as CD11b43 or CD11a, CD18, and VLA–4,49 which mediate the stable adhesion of leukocytes to activated endothelium. Third, as mentioned above, statins directly block the LFA–1–intercellular adhesion molecule-1 interaction41 that occurs during leukocyte extravasation to sites of inflammation. Finally, via RhoA inactivation, statins inhibit actin polymerization in monocyctic cells,39 thus potentially disturbing integrin-dependent leukocyte adhesion, a process known to be modulated by cytoskeletal organization.44,45

In summary, the present study demonstrates that fluvastatin inhibits vascular TF expression and macrophage accumulation even in the absence of plasma cholesterol lowering. This finding extends previous observations indicating that lowering plasma cholesterol levels by statin treatment reduces the atherothrombotic profile of experimental lesions21–27 and supports the hypothesis of a potential synergism between cholesterol-dependent and cholesterol-independent properties of statins,18 with both contributing to plaque stabilization and prevention of thrombotic complications.

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