Dietary Lipid Lowering Modifies Plaque Phenotype in Rabbit Atheroma After Angioplasty
A Potential Role of Tissue Factor

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Background—Tissue factor (TF), the main initiator of blood coagulation, is involved in cellular migration and angiogenesis processes. TF is expressed strongly in lipid-rich plaques and probably plays an important role in the thrombotic complications of plaque rupture. This study analyzes the effect of dietary lipid lowering on TF expression and cellular modifications in angioplasty-induced rabbit plaque rupture.

Methods and Results—After experimental plaque rupture by balloon angioplasty in atheromatous rabbits, animals were assigned a 0.2% or a 2% cholesterol diet, and the TF content of arterial wall and the associated histological modifications were analyzed after 4 weeks. Early effects of lipid lowering were observed: The increase of TF expression in the vascular wall was stronger in the 2% than in the 0.2% cholesterol diet group (iliac arteries: 1226±308 versus 72±29 mU TF/g artery, \( P<0.005 \)). Immunohistochemistry indicated that TF expression was associated with sprout of neovessels, which was more pronounced in the 2% than in the 0.2% cholesterol group.

Conclusions—This study shows that dietary lipid lowering decreases the thrombotic potential of ruptured atherosclerotic plaques through TF decrease. Moreover, high TF expression is associated with marked angiogenesis in the vascular wall, which is reduced by lipid lowering. These results provide further arguments for strong dietary lipid lowering to reduce plaque instability and thrombogenicity. (Circulation. 2003;108:1740-1745.)

Key Words: tissue factor ▪ atherosclerosis ▪ angioplasty ▪ lipids

Triggering of thrombosis by plaque disruption is probably the most important mechanism leading to the onset of acute arterial disease and ischemic sudden death. One of the most powerful actors in plaque thrombogenicity is tissue factor (TF), the main initiator of blood coagulation in vivo. TF is a membrane-bound procoagulant molecule that acts as an essential cofactor to factor (F) VII/VIIa leading to thrombin generation.1 Moreover, it was recently demonstrated that TF has additional biological functions as an immediate early gene and plays an important role in cell migration and in angiogenesis.2,3

In normal arteries, TF antigen and mRNA are detectable in adventitia fibroblasts but not in the media and in the endothelial cells.4,5 In human atherosclerotic specimens from endarterectomy or coronary atherectomy, active TF is detectable in macrophages, mesenchymal intimal cells, and the extracellular matrix.6,7 It was recently demonstrated that human plaque thrombogenicity correlated with the TF content of the plaque.8 In human pathological lesions, it has been shown that the TF content of de novo lipid-rich plaques is higher than that of stenotic fibrous plaques.9 Lipid-lowering strategies are beneficial in the onset of cardiovascular events, probably through a decreased plaque instability and thrombogenicity. At this time, little is known about the relationships between TF expression and cellular or molecular alterations in atheromatous plaques after lipid lowering.

Rabbit models have been widely used because they rapidly develop atherosclerotic lesions on a high-cholesterol diet.9,10 These rabbit lesions are characterized by a higher content of lipids and macrophages than the human lesions. Balloon injury accelerates atheroma formation and makes lesions more uniform in size and distribution.11 Such lesions resemble the so-called “vulnerable” human atheroma more closely than the typical foam-cell lesions in rabbits produced by hypercholesterolemia alone. TF antigen and activity are present in rabbit plaques, as demonstrated by immunohistochemistry, specific binding of labeled FVIIa, or chromogenic assays.9,12,13

The aim of the present study was to examine the effect of 2 different cholesterol diets on TF expression and on vascular lesion development in a rabbit model of plaque rupture.
Methods

Male New Zealand White rabbits (Charles River, Saint Aubin les Elbeuf, France) with an initial body weight of 3.0 to 3.5 kg were used for this study (5 control rabbits, 14 hypercholesterolemic rabbits). All experiments were conducted in compliance with the position of the American Physiological Society on research animal use. The study protocol is shown in Figure 1.

Induction of Atherosclerosis

The 14 rabbits were anesthetized with ketamine (0.5 mL/kg Ketalar, Parke-Davis) and xylazine (0.25 mL/kg Rompun, Bayer), 1 intravenous infusion. A 3F Fogarty balloon catheter (Baxter) was introduced via a femoral arteriotomy, was inflated and pulled back through the aorta and the iliac arteries, thereby causing endothelial denudation. The procedure was repeated 3 times and was performed on each iliac artery. All animals were placed on a rabbit chow diet containing 2% cholesterol.

Angioplasty

Six weeks after atherosclerosis induction, rabbits were anesthetized as described above. Through a midline neck incision, the carotid artery was isolated. Through an arteriotomy, a guidewire was introduced and positioned into the iliac artery. After angiography, the 14 rabbits were anesthetized with ketamine (0.5 mL/kg Ketalar, Stago), and 25 μL FVIIa (50 nmol/L, Stago). One milliliter of CI+ (3 IU/mL, Biogenic) was then rapidly added, and the reaction product was measured, over a period of 10 minutes at 405 nm, in an ELISA plate reader (Labsystems). A standard curve was created with a rabbit thromboplastin (CI+, Stago). One milliliter of CI+ was defined as having 1000 U of TF activity. TF activity was expressed as mU TF/g artery.

Assays on Plasma Samples

Blood samples were collected immediately before euthanasia to study plasma lipid parameters. Serum total cholesterol and triglyceride levels were determined with enzymatic assays using cholesterol esterase plus cholesterol oxidase and glycerol-3 phosphate oxidase, respectively (Biomerieux).

Euthanasia and Specimen Preparation

Ten weeks after the initial injury, all animals were killed with an overdose of pentobarbital. An immediate abdominal cutdown was performed, and abdominal aorta and iliac arteries were removed. Three 5-mm sections were taken from abdominal aorta and iliac arteries, kept in 4% paraformaldehyde in 30% sucrose solution, embedded in OCT (methylmethacrylate) compound, frozen in liquid nitrogen, and stored at −80°C. The samples were cryosectioned (7 μm) onto SuperFrost slides and kept at −80°C until immunohistochemistry.

After 1 night in 30% sucrose solution, the remaining sections without adventitia (mechanically eliminated) were stored immediately at −80°C before being used for arterial TF activity assay.

Arterial TF Activity Assay

The remaining arteries were weighed and mixed with extraction buffer (Tris-HCl 20 mmol/L, Nonidet NP40 0.5%, aprotinin 2 mg/mL, benzamidin 5 mmol/L, amino caproic acid 100 mmol/L, sodium azide 0.1%). Samples were homogenized at 24 000 rpm for 1 minute with a high-speed homogenizer (Ultra Turax T25, Ika-Labotechnik) and sonicated at 2.5 kHz for 30 seconds. After 1 hour at 4°C, specimens were centrifuged at 3300 g for 15 minutes. Supernatants were stored at −80°C.

TF activity was analyzed by a chromogenic FXa generation assay. Supernatants (25 μL) were incubated for 3 hours at 37°C with 25 μL HEPES BSA buffer, 25 μL FVIIa (50 nmol/L, Stago), 25 μL FX (750 nmol/mL, Stago), and 25 μL CaCl2 25 mmol/L. The mixture was centrifuged for 3 minutes at 3300 g at 4°C. Aliquots (50 μL) were removed from the reaction mixture and placed in a 96-well microtiter plate preloaded with 125 μL of EDTA 0.05 mol/L. Chromogenic substrate S2765 (25 μL, Biogenic) was then rapidly added, and the reaction product was measured, over a period of 10 minutes at 405 nm, in an ELISA plate reader (Labsystems).

The amidolytic activity was characterized as TF according to a neutralization procedure using a monoclonal anti-rabbit TF antibody (AP-1).

In defined experiments, supernatants were incubated in absence of FVIIa and FX.

Culture and TF Activity of Mononuclear Cells

The mononuclear cells were isolated by gradient centrifugation as described previously.14 Cell preparations (3×10⁶ cells/mL) suspended in RPMI 1640 without FCS were cultured for 16 hours without or with stimulation by endotoxin at 5000 EU/mL (Escherichia coli 055:B5; Sigma Chemical); these are referred to as unstimulated and stimulated cells, respectively.

TF activity was determined in lysed cells with a modified amidolytic assay with prothrombin concentrate complex (Laboratoire de Fractionnement et des Biotechnologies) as a source of FVII (3 IU/mL). Results were expressed as mU of TF/1000 monocytes. The amidolytic activity was characterized as TF according to a neutralization procedure using AP-1.

Immunohistochemistry

Immunohistochemistry was performed with AP-1 and revealed with Vectastain Elite ABC peroxidase Kit (Vector). A negative control
(sample without primary antibody incubation) was included. TF antibody was diluted (1/50) in PBS and applied for 1 night at 37°C in a humidified chamber. Color was developed for peroxidase by diaminobenzidine.

Antibodies against endothelial cells (anti-CD31, Dako), macrophages (anti-RAM11, Dako), and smooth muscle cells (SMCs) (anti-α-actin, Boehringer) were used, according to the same procedure, to stain artery constitutive cells and microvessels in adjacent serial sections.

Statistical Analysis
Results are expressed as mean±SEM. Data were analyzed by use of a nonparametric test (Kruskal-Wallis) to determine significant differences (P<0.05) between groups, followed by the Mann-Whitney U test to test the significance of differences between groups.

Results
Lipid Parameters
Plasma cholesterol levels were increased in all the cholesterol-fed rabbits compared with the control group (37±3 mg/dL; P<0.001). Overall, in the 0.2% cholesterol group, the cholesterol level was 25% lower than in the 2% cholesterol group (1574±266 and 2094±200 mg/dL, respectively, P=0.16). No significant difference was observed in the triglyceride levels between the 2 cholesterol-fed rabbit groups and the control group (126±28 and 192±80 mg/dL, respectively, compared with the control group, 108±17 mg/dL; P=0.56).

Arterial TF Activity
All artery extracts expressed significant coagulant activity that was neutralized by an anti-TF antibody. No coagulant activity was found in specimens tested without purified FVIIa and FX. Results are shown in Figure 2.

In the 0.2% cholesterol group, TF activity was significantly higher than in the control group (4±2 mU TF/g artery), but this increase was significant only in the iliac arteries (P<0.05). Moreover, TF activity was higher in the iliac arteries than in the aorta (72±29 versus 18±5 mU TF/g artery, P<0.05).

In the 2% cholesterol group, TF activity was increased dramatically both in iliac arteries and in the aorta compared with the control group (P<0.01 and P<0.05, respectively). Moreover, TF activity was significantly higher in the iliac arteries than in the aorta (1226±308 versus 149±51 mU TF/g artery, P<0.05).

Overall, in the 2% cholesterol group, TF activity was 8-fold higher (P<0.05) in the aorta and 17-fold higher (P<0.005) in the iliac arteries than in the 0.2% cholesterol group.

Monocyte TF Activity
Neutralization assay with TF antibody confirmed that the amidolytic activity measured was TF in all cases. Results are shown in Figure 3.

In the 2 hypercholesterolemic groups, TF activity was increased in both unstimulated and stimulated cells compared with the control group (P<0.05). TF activity was significantly higher in the 0.2% cholesterol group than in the 2% cholesterol group in both unstimulated and stimulated cells (P<0.05).

Immunohistochemistry
In atherosclerotic arteries, the neointimal development was larger in the iliac arteries, which underwent angioplasty, than in the aorta. The neointima was larger in the 2% than in the 0.2% cholesterol group. Large acellular areas, probably corresponding to lipid-loaded zones, were observed only in the 2% cholesterol group.

Four weeks after angioplasty, iliac and aortic lesions contained macrophages and a positive SMC layer, with similar distributions. Macrophages, intimal SMCs, and endothelial cells stained positively for TF. The media underlying atheroma also stained for TF. TF staining was more pronounced in the iliac arteries than in the aorta. Moreover, in the 2% cholesterol group, TF staining was more intense than in the 0.2% cholesterol group (Figure 4).
In the 2% cholesterol group, immunostaining with specific antibodies against endothelial cells showed large vessels in the media and the neointima. In the 0.2% cholesterol group, only few and thin vessels were observed in the subadventitial zone (Figure 5).

Discussion

In the present study, we compared TF expression 4 weeks after angioplasty in an atherosclerotic rabbit model according to 2 cholesterol diets (0.2 and 2%). The results confirm that TF activity is enhanced 4 weeks after angioplasty in the circulating monocytes and in the arterial wall. Moreover, the increase in cholesterol content of the diet induces a strong increase of TF activity in the vascular wall, particularly in the iliac arteries, which underwent angioplasty. Interestingly, cholesterol diet differently affects the TF expression in circulating monocytes, because the TF response decreases when the cholesterol content of the diet increases. Conversely, our data suggest an impact of lipid lowering on neovascularization, possibly via TF. This hypothesis is further strengthened by our observation of numerous microvessels from the rabbit with the highest-cholesterol diet.

Several previous studies demonstrated the importance of TF on the thrombogenicity of atheromatous plaques. In the thoracic aorta of rabbits that had undergone a single balloon injury associated with atherogenic diet (0.3% cholesterol, 4.7% coconut oil) for 4 months, Aikawa et al observed a strong induction of TF expression and activity. In this model, TF expression was expressed strongly in macrophages and was also present in the SMCs and in the endothelial cells. The return to a normal diet (for 16 months) led to a decrease of TF content of the vascular wall.

Our model differs from that of Aikawa et al in several aspects: (1) balloon angioplasty at 6 weeks that mimics plaque rupture, (2) lipid diet after angioplasty, and (3) simultaneous analysis of TF activity in the vascular wall and in circulating monocytes. After the second injury, one group of rabbits remained on 2% cholesterol diet, and another group was fed a 0.2% cholesterol diet. Four weeks after angioplasty, a 25% decrease of serum cholesterol was observed in the 0.2% compared with the 2% cholesterol group. At this time, a marked increase in TF expression was observed in the arterial wall of all hypercholesterolemic animals. Endothelial cells, neointimal macrophages, and SMCs expressed TF antigen. Our results are consistent with those of Gertz et al in femoral arteries. Interestingly, TF activity was higher in iliac arteries, which underwent the double injury, than in the aorta, indicating that plaque rupture is indeed a major trigger for TF induction, probably through mechanical injury and perhaps sustained by an ongoing inflammatory process. It is known that oxidized lipids and free cholesterol can directly induce TF expression in macrophages. The effect of lipids

Figure 3. Effect of vessel injury and cholesterol diet on monocyte TF activity. Open bars indicate mononuclear cells cultured without endotoxin; solid bars, mononuclear cells cultured with endotoxin (5000 EU/mL). Results are expressed as mU TF/1000 monocytes for control (n=5), 0.2% cholesterol (n=7), and 2% cholesterol (n=5) groups. *P<0.05 vs control group; †P<0.05 vs 0.2% cholesterol group in stimulated and unstimulated cells, respectively.

Figure 4. Effect of vessel injury and cholesterol diet on TF expression. Representative photomicrographs show sections of iliac artery of 2% cholesterol (A) and 0.2% cholesterol (B) groups. In 2% cholesterol group, an important acellular zone was present (arrow), and TF staining was more intense than in 0.2% cholesterol group. TF was identified in frozen sections by AP-1. Hematoxylin counterstaining. Magnification ×50.
could also be explained by the induction of proinflammatory cytokines from local arterial cells. Atheroma cells produced cytokines and growth factors, which can amplify vascular cell activation.

In our study, we observed modification of plaque phenotype by lipid lowering. First, neointimal hyperplasia was more significant in the 2% than in the 0.2% cholesterol group. In the highest-cholesterol group, a large part of the neointima consisted of an acellular zone, probably corresponding to lipids. The cellular part consisted of macrophages organized in “clusters,” and a SMC layer overlay the atheromatous core. In the 0.2% cholesterol group, the neointima was more cellular and consisted of SMCs and disseminated macrophages.

Recent experimental studies have demonstrated that lipid lowering by diet alone or by treatment reduces macrophage accumulation and SMC death within the aortic lesions of hypercholesterolemic rabbits.11,19,20 In these studies, all rabbits were killed 16 months after diet modification. In our model, cellular modifications were already observed 4 weeks after angioplasty associated with diet modification, providing intermediate data in a model of updated injury. Moreover, cell distribution remains similar between the iliac and aortic segments. This suggests that the increase in TF activity observed in the iliac arteries compared with the aorta is related to activation of TF expression rather than to macrophage enrichment. This finding provides further information on the sequence of events after plaque modifications after angioplasty.

Interestingly, we observed more neovessels in the arterial wall in the 2% cholesterol group than in the 0.2% cholesterol group. Overall, the morphology of these vessels was different. In the 0.2% cholesterol group, we observed organized endothelial cells in the adventitial and the medial layer. In the 2% cholesterol group, large vessels were observed, and some of them were irregularly shaped. It has been suggested that intraplaque neovascularization of coronary artery atherosclerotic plaques may be important in the growth of these lesions. One possible mechanism could be that neovessels were the vehicle for delivering inflammatory cells and macrophages. In humans, McCarthy et al21 had demonstrated that angiogenesis is increased significantly in carotid artery atherosclerotic plaques from symptomatic patients compared with matched individuals with asymptomatic plaques. It becomes evident that atherosclerosis progression shares several features with cancer progression and metastasis, including the development of microvessels within atherosclerotic plaques.22 In addition to its well-established role in blood coagulation, TF is associated with various other physiological processes, such as inflammation, angiogenesis, and metastasis. It has become clear that TF–FVIIa interaction elicits a variety of intracellular signaling events that may be implicated in these actions.23 In several models, TF expression mediates upregulation of the proangiogenic vascular endothelial growth factor (VEGF)24 that can act directly on endothelial cells to promote vessel formation. Makin et al25 recently established that plasma levels of TF and VEGF are increased in patients with peripheral artery disease. Moreover, it was recently demonstrated that lipid-lowering therapy can reduce VEGF in hypercholesterolemic patients.26 This could be responsible for decreased angiogenesis in the atherosclerotic plaques. By its pleiotropic effects, TF could promote plaque instability by both increased thrombogenicity and neovascularization.

In our model, we also tested the relationships between the dietary lipid content and monocyte TF expression. First, we confirmed our previous results indicating that angioplasty enhances TF response to endotoxin in circulating monocytes.14 However, the effect of the lipid content of the diet on circulating monocytes is different from that in the vascular wall: The decrease in cholesterol content of the diet did not induce a decrease of monocyte TF response but rather an increase. This agrees with a previous study from our group indicating that monocytes from hyperlipidemic subjects exhibited a depressed response to endotoxin.27 These results suggest that the capacity of circulating monocytes to express TF in response to stimulation is reduced when plasma atherogenic fractions increase. Moreover, monocytes from

Figure 5. Effect of vessel injury and cholesterol diet on neovascularization. Vessels were identified by antibody against endothelial cell marker CD31. A and B, in 2% cholesterol group, numerous large vessels were shown in vessel wall. C and D, in 0.2% cholesterol group, only few and thin vessels were observed. Hematoxylin counterstaining. Magnification: A and C, ×50; B and D, ×100.
hypercholesterolemia have altered characteristics and functions. Hypercholesterolemia induces monocyte adhesion, penetration, and transformation into foam cells in the vascular wall. Conceivably, dietary lipid lowering could promote a decreased penetration of the most activated monocytes in the vascular wall, leading to an apparent increase of the monocyte response to external stimuli.

The extrapolation of data from any animal model to human atherosclerosis requires caution. However, the rabbit model we used has been demonstrated to have several features in common with human atherosclerosis and to be reproducible.21-25 The combination of initial balloon injury with high-cholesterol diet results in severe atherosclerotic lesions and very high cholesterol levels and is associated with a significant morbidity rate in the animals. This model produces lesions that resemble those in humans more closely than lesions produced by hypercholesterolemia alone.

The present study confirms and extends previous ones on the mechanisms by which dietary lipid lowering decreases thrombotic potential and neovessel formation in atherosclerotic plaques after rupture. Deep-vessel injury secondary to plaque rupture or ulceration results in exposure of substrates that may lead to persistent thrombotic occlusion. Lipoproteins or their derivatives, together with mechanical plaque rupture, promote both local and general inflammation through various mechanisms, including atheroma-related growth factor, inflammatory cytokines, and macroinfiltration by neovessels. These results provide further arguments for strong lipid lowering to reduce thrombogenicity and neovascularization of plaques.

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References

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