Dietary Lipid Lowering Reduces Tissue Factor Expression in Rabbit Atheroma

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**Background**—The mechanisms by which lipid lowering reduces the incidence of acute thrombotic complications of coronary atheroma in clinical trials remains unknown. Tissue factor (TF) overexpressed in atheroma may accelerate thrombus formation at the sites of plaque disruption. A cell surface cytokine CD40 ligand (CD40L) enhances TF expression in vitro.

**Methods and Results**—To test the hypothesis that lipid lowering reduces TF expression and activity, we produced atheroma in rabbit aortas by balloon injury and cholesterol feeding for 4 months (Baseline group, n = 15), followed by either a chow diet (Low group, n = 10) or a continued high-cholesterol diet for 16 months (High group, n = 5). Immunolocalization of TF, CD40L, and its receptor CD40 was quantified by computer-assisted color image analysis. Macrophages in atheroma of the Baseline and High groups strongly expressed TF. Intimal smooth muscle cells and endothelial cells also contained immunoreactive TF. Regions of expression of CD40L and CD40 colocalized with TF. Protein expression of TF diminished substantially in the Low group in association with reduced expression of CD40L and CD40. In situ binding of TF to factors VIIa and X, detected by digoxigenin-labeled factors VIIa and X, colocalized with TF protein in atheroma and decreased after lipid lowering. We also determined reduced TF biological activity in the Low group by use of a chromogenic assay. The level of TF mRNA detected by reverse transcription–polymerase chain reaction also decreased after lipid lowering.

**Conclusions**—These results suggest decreased expression and activity of TF as a novel mechanism of reduced incidence of thrombotic complications of atherosclerosis by lipid lowering. (Circulation. 1999;100:1215-1222.)

**Key Words:** atherosclerosis ■ thrombosis ■ hypercholesterolemia ■ coagulation ■ macrophages

We now recognize that thrombus formation causes most acute coronary syndromes, including unstable angina and myocardial infarction.1–4 The thrombogenicity of atheroma results in large measure from overexpression of the potent procoagulant tissue factor (TF).5–7 This membrane-bound glycoprotein initiates the blood coagulation cascade by binding with and enhancing the enzymatic activity of coagulation factor VIIa to its substrates factors IX and X.8–10 TF in atheroma may accelerate thrombus formation at the sites of atherosclerotic plaque disruption when the procoagulant within the plaque comes in contact with blood. Thus, the TF content of the atheromatous lesion is 1 key determinant of the acute coronary syndromes. Indeed, the culprit lesions of patients with acute coronary syndromes contain higher levels of TF protein than do those from stable atheroma.11–14 Recent clinical trials have demonstrated that lipid lowering by diet reduces macrophage accumulation, reduces proteinase expression and activity, and increases collagen content in atheroma of hypercholesterolemic rabbits, features that should reinforce the resistance of fibrous cap to rupture.15–19 These benefits do not appear to accrue so much from improvement in luminal caliber as from functional changes in the atheroma itself that are commonly called stabilization. However, the precise molecular and cellular mechanisms that underlie this lesion stabilization that leads to the striking clinical benefit remain speculative. We have demonstrated that lipid lowering by diet reduces macrophage accumulation, reduces proteinase expression and activity, and increases collagen content in atheroma of hypercholesterolemic rabbits, features that should reinforce the resistance of fibrous cap to rupture.20 We also have recently reported that dietary lipid lowering promotes accumulation of more mature smooth muscle cells (SMCs) in the fibrous cap of plaque.21 However, to date, no study has addressed the crucial issue of the thrombogenicity of plaque in relation to lipid lowering. Atheroma disruption by itself would have little clinical consequence were it not for inciting thrombus formation.
Hence, this study tested the hypothesis that lipid lowering by diet reduces expression and activity of TF in atheroma of hypercholesterolemic rabbits.

Recent work from our laboratory identified interaction of CD40 ligand (CD40L, CD154) and its receptor CD40, an inflammatory signaling dyad found in human atheroma, as potentially the key trigger in TF expression by macrophages, the cell type most responsible for TF overexpression in atheroma. Therefore, we further tested the hypothesis that lipid lowering by diet reduces expression of CD40L and CD40 in the atheromata of these rabbits as a possible mechanism of any attenuation of TF. Our results shed considerable new light on the regulation of the thrombogenicity of the atherosclerotic plaque and provide new evidence regarding the mechanisms whereby lipid lowering can reduce the thrombotic complications of atheroma, such as unstable angina or myocardial infarction.

Methods

Animal Experimental Protocol and the Diet

Thirty New Zealand White male rabbits were fed an atherogenic diet for 4 months to create atheroma. Balloon injury by Fogarty embolectomy catheter of thoracic aortas was performed 1 week after initiation of atherogenic diet. Fifteen rabbits killed at 4 months composed Baseline group. Five animals continued atherogenic diet (High group), and remaining animals consumed a chow diet with no added cholesterol and fat for 16 months (Low group).

Plasma Cholesterol and TG Levels

Peripheral blood was collected from the ear artery under local anesthesia for measurement of plasma total cholesterol (TC) and triglyceride (TG) concentrations by enzymatic assays (Sigma).

Tissue Preparation

Rabbits were euthanized with sodium pentobarbital (120 mg/kg IV). The proximal portion of the thoracic aorta (2 mm below the ligamentum arteriosum) was excised and snap-frozen with OCT compound (Sakura Finetek Inc) for fresh-frozen sections. The rest of the thoracic aorta for detection of TF mRNA and activity was quickly frozen with liquid nitrogen.

Immunohistochemistry

Monoclonal antibodies used in this study are as follows: mouse monoclonal antibodies against rabbit TF (No. 4510, American Diagnostica Inc), rabbit CD11b (Spring Valley Laboratories), human α-smooth muscle actin (1A4, Dako Corp), and human CD40 (MCA679, Serotec Ltd) and rat monoclonal anti-mouse CD40L (M158, a gift of ImmuneX). Immunohistochemistry was performed on fresh-frozen sections (6 μm) by the standard ABC method (Vector) as described in our recent articles regarding other aspects of the same animals. The percentages of immunopositive intimal areas were measured with the Optimas 5.2 image analysis system. The statistical testing used 1-way ANOVA followed by Fisher’s test. Linear regression analysis was performed with the absolute positive areas of TF, CD40L, and CD40 staining.

In Situ Binding Assay of TF to Coagulation Factors VIIa and X

Binding of TF to factors VIIa and X was detected in situ by use of digoxigenin-labeled factors VIIa and X (DigVIIa and DigX) as previously described. For detection of in situ DigVIIa binding, fresh-frozen sections were fixed with acetone at −20°C for 10 minutes and incubated with 5 mmol/L DigVIIa in Tris-buffered saline (pH 7.5) containing 5 mmol/L CaCl2 at 37°C for 1 hour. Sections were treated with 4% paraformaldehyde, incubated with a sheep Fab anti-digoxigenin antibody conjugated with horseradish peroxidase at 37°C for 1 hour, and then incubated with 3,3′-diaminobenzidine for 10 minutes. Unlabeled factor VIIa was applied instead of DigVIIa as a negative control. For DigX binding, sections were incubated with 10 mmol/L recombinant factor VIIa for 1 hour and then incubated with 10 mmol/L DigX at 37°C for 3 hours. Sections were fixed with 4% paraformaldehyde and stained with anti-digoxigenin antibody as mentioned above. DigX staining was also performed without added recombinant factor VIIa as a negative control. Immunohistochemistry for rabbit TF on serial sections was also performed to determine colocalization of protein expression and in situ binding of TF.

TF Activity Assay

TF activity was determined by chromogenic measurement of generation of the factor Xa on total tissue lysates. Frozen aortas were homogenized with 50 mmol/L Tris, 100 mmol/L NaCl, and 1% BSA, pH 7.6 (Tris buffer) on ice and centrifuged at 10 000 g at 4°C for 15 minutes. Pellets were reconstituted with Tris buffer. Lysates were incubated at room temperature with or without anti-rabbit TF antibody (No. 4510, American Diagnostica) for 30 minutes. Human factor VIIa, factor X, and the chromogenic substrate (Spectrozyme Xa, American Diagnostica) were added, and anti-rabbit TF antibody–inhibitable values were measured. Optical density was measured at 410 nm, and total protein was measured by BCA Kit (Pierce). Arterial TF activity was quantified by reference to a standard curve constructed with recombinant human TF (No. 4500L, American Diagnostica), and 30 ng/mL of this protein yields a clotting time of 30 seconds. TF activity with a 30-second clotting time was defined as 1 U/mL, and arterial TF is expressed as mU/100 μg protein.
RNA Extraction and Reverse Transcription–Polymerase Chain Reaction

Total RNA of aortas from 3 groups was extracted by the acid guanidinium thiocyanate–phenol-chloroform method. Total RNA of rabbit peritoneal macrophages elicited by lipopolysaccharide (1 mg/mL) was used as a positive control. Two pairs of primers were designed to detect rabbit TF mRNA: sense, 5′-AAGCAGTGA-3′; antisense, 5′-AACACACATGGGACGAG-3′) and rabbit G3PDH as an internal control: sense, 5′-GGAGCCAAAAGGTCATC-3′; antisense, 5′-CCAGTGAGTTTCCCGTTC-3′. Each polymerase chain reaction (PCR) cycle consisted of denaturing at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and elongation at 72°C for 60 seconds. PCR for TF and G3PDH was conducted for 40 cycles, and products were electrophoresed on 2.0% agarose gel.

Results

Plasma Lipid Values

At the beginning of the experiment (n=30), the mean TC and TG levels (mg/dL) were 43±4 and 52±14, respectively, and rose to 1562±123 and 244±49 after 4 months on the atherogenic diet. The TC level returned to baseline after 16 months on the control diet lacking supplemental lipids (Low group [n=10]; TC, 19±3; TG, 58±10) but remained elevated in the High group (n=5; TC, 1108±158; TG, 224±87).

All Cell Types in Rabbit Atheroma Express TF and Its Potent Inducers (CD40 Ligand and CD40)

Immunohistochemistry was performed to localize TF protein in rabbit atheroma by use of the monoclonal antibody against rabbit TF. In the Baseline group, after 4 months of atherogenic diet, the aortic lesions contained prominent macrophages (identified by anti-CD11b antibody) underlying a SMC layer (identified by anti-α-smooth muscle actin antibody) like fibrous cap of human plaques. TF is expressed predominantly by macrophages in atheroma. Intimal SMCs and ECs also expressed TF. Staining of TF was found in adventitia as well. Arrowhead indicates internal elastic lamina. Bottom left and middle, Immunoreactive CD40L and CD40 colocalized with TF protein in atheroma. Scale bar=200 μm. Magnification ×100. Bottom right, Double immunostaining for TF and CD11b demonstrates TF expression by SMCs and macrophages at higher magnification. Localization of TF protein stained red and CD11b stained blue. Macrophages expressing both TF and CD11b show purple color (arrow). Spindle-shaped cells with red are TF-positive SMCs. Scale bar=50 μm. Magnification ×400.

Figure 2. All vascular cells express TF in rabbit atheroma after 4 months of atherogenic diet (Baseline lesions). Top 3 panels, Atheroma contained prominent macrophage (Mφ) accumulation (detected by anti-CD11b antibody) underlying a SMC layer (identified by anti-α-smooth muscle actin antibody) like fibrous cap of human plaques. TF is expressed predominantly by macrophages in atheroma. Intimal SMCs and ECs also expressed TF. Staining of TF was found in adventitia as well. Arrowhead indicates internal elastic lamina. Bottom left and middle, Immunoreactive CD40L and CD40 colocalized with TF protein in atheroma. Scale bar=200 μm. Magnification ×100. Bottom right, Double immunostaining for TF and CD11b demonstrates TF expression by SMCs and macrophages at higher magnification. Localization of TF protein stained red and CD11b stained blue. Macrophages expressing both TF and CD11b show purple color (arrow). Spindle-shaped cells with red are TF-positive SMCs. Scale bar=50 μm. Magnification ×400.
but not for CD11b. All vascular cell types in the intima of the baseline lesion contained CD40L and CD40, both of which colocalized with TF. Negative controls, in which nonimmune mouse IgG or PBS was applied in place of the specific monoclonal antibodies, abrogated the staining (data not shown).

**TF Expression Decreases in Atheroma During Lipid Lowering in Association With Reduced Expression of CD40L-CD40**

After 16 months of continued high-cholesterol diet, high levels of TF, CD40L, and CD40 expression persisted, predominantly in macrophages but in intimal SMCs and ECs as well (Figure 3). However, expression of TF, CD40L, and CD40 by macrophages decreased in the intima of the Low-group animals after 16 months of dietary lipid lowering in association with a reduced number of macrophages (Figure 4). SMCs and ECs also exhibited decreased expression of TF, CD40L, and CD40.

Quantitative color image analysis substantiated significant reduction in TF, CD40L, and CD40 expression in the intima during lipid lowering (Figure 5). To test the association of CD40L-CD40 and TF, we determined the correlation between the immunopositive areas for these molecules (measured in square millimeters). Linear regression analysis revealed a high correlation of the amount of plaque area positive for CD40L and CD40 and that occupied by TF ($R^2=0.8003$ and $R^2=0.8047$, respectively) (Figure 6).

**Lipid Lowering Also Decreases Functional Activity of TF in Plaques**

In addition to assessing the presence of immunoreactive TF protein, we sought evidence of TF function by evaluating the in situ binding of the TF interactors, the coagulation factors VIIa and X, detected by DigVIIa and DigX (Figure 7). DigVIIa and DigX colocalized with TF protein in atheroma of the Baseline and High groups. However, DigVIIa and DigX binding decreased in the intima of the Low-group animals, whereas the adventitia stained positively, as expected. Similar results were obtained from 5 animals from each group. A factor VIIa binding assay using unlabeled factor VIIa and a DigX binding assay without added recombinant factor VIIa showed no staining (data not shown).

We also determined enzymatic activity of TF using a chromogenic assay that detects generation of factor Xa by cleavage of factor X by TF (Table). TF activity was detected in extracts of all aortas studied. However, lipid lowering by diet produced statistically significant reduction in arterial TF activity in aortic extracts from the Low group.

**Figure 3.** Expression of TF, CD40L, and CD40 in rabbit atheroma at 16 months of continued hypercholesterolemia (High group). Top 3 panels, TF expression predominantly in lesional macrophages (CD11b) persisted in atheroma of High group. SMCs and ECs also displayed TF expression. Bottom panels, CD40L and CD40 colocalized TF expression in intima. Arrowhead indicates internal elastic lamina. Scale bar = 200 μm. Magnification ×100.
Lipid Lowering Reduces TF mRNA Expression in Aortas of Hypercholesterolemic Rabbits

Reverse transcription (RT)-PCR was performed to determine whether lipid lowering reduces TF expression at mRNA level in aortas of hypercholesterolemic rabbits. TF mRNA (254 bp) was detected in rabbit peritoneal macrophages elicited by lipopolysaccharide and aortas from both the Baseline and High groups (Figure 8). However, expression levels of TF mRNA decreased substantially in the Low group after 16 months of dietary lipid lowering, whereas G3PDH mRNA expression (346 bp) was similar in all 3 groups. PCR products were not detected on all samples without RT reaction.

Discussion

TF probably plays an important role in the pathogenesis of thrombus formation at sites of atheromatous plaque disruption, resulting in acute coronary events such as unstable angina or myocardial infarction. Wilcox et al and Drake et al reported TF expression in human atheroma and adventitial fibroblast-like cells. Thiruvikraman et al localized TF in situ using DigVIIa and DigX in all vascular cell types in human atherosclerotic plaques and in experimental arteriosclerosis in rat, rabbit, and pig. Hatakeyama et al reported a similar pattern of TF expression in human aorta. Marmur et al detected TF activity in human coronary atheroma. Several recent studies have demonstrated increased expression of TF by lesional macrophages in atherectomy specimens obtained from patients with acute coronary syndromes. These various lines of evidence strongly support the principal role of TF in determining the thrombogenicity of atheromata and hence their propensity to provoke acute complications. Accumulating evidence has established that lipid lowering reduces coronary events and mortality in patients. One mechanism that may underlie such clinical benefits of lipid lowering might be reduced expression of proteins, including TF, that may accelerate thrombus formation at the sites of plaque rupture. The present study demonstrates that lipid lowering by diet indeed reduces expression of TF by cells within rabbit atheroma.

In the rabbits studied here, as in human atheroma, TF expression was detected predominantly on lesional macrophages in the Baseline and High groups by immunohistochemistry using anti-rabbit TF antibody. Some SMCs and ECs in atheroma also exhibited TF expression. To elucidate the state of TF identified by the antibody, we performed in situ binding assays for factors VIIa and X. These studies show that virtually all of the areas identified as containing TF antigen also bound factors VIIa and X. In addition, binding of factor X occurred only in the presence of VIIa. This suggests that the TF antigen contains an intact active site and thus is potentially active. In addition, we determined that TF activity generates factor Xa by cleaving factor X. Both protein expression and activity of TF decreased substantially during lipid lowering. Increased expression of TF and enhanced procoagulatory activity by monocytes/macrophages incubated with modified lipoproteins or free cholesterol have previously been reported. Lipid lowering may reduce lipid accumulation within atheroma and in turn decrease TF expression and activity.

A number of molecular mediators, including the atheroma-related growth factors or inflammatory cytokines, CD40L...
(CD 154), platelet-derived growth factor (PDGF), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β), may modulate TF expression in macrophages, SMCs, or ECs. Increased production of such mediators in atheroma should enhance TF expression in an autocrine or paracrine manner. In this study, immunoreactive CD40L and CD40 colocalized well with regions of TF protein expression in atheroma, compatible with a role for CD40 ligation in inducing TF expression in vitro. Lipid lowering markedly reduced CD40L-CD40 expression within the rabbit atheroma, illustrating a possible mechanism mediating the concomitant fall in TF levels. Decreased expression of other cytokines also probably contributes to reduced TF expression by cells in atheroma during lipid lowering. In this study, expression of TF mRNA decreased in aortas after lipid lowering. Intracellular regulation of TF gene expression involves certain transcription factors, such as AP-1 and nuclear factor (NF)-κB. Activated NF-κB colocalizes with TF within atheroma. Further experiments are required to determine whether dietary lipid lowering interrupts the action of transcription factors that regulate genes that contribute to plaque disruption or thrombus formation.

The reduction in TF reported here in part reflects diminished macrophage population during lipid lowering. This decrease in macrophage accumulation may have several molecular and cellular bases, analogous to those that regulate TF expression itself. For example, lipid lowering may reduce the local production of inflammatory cytokines, such as TNF and IL-1, which augment expression of leukocyte adhesion molecules on ECs, as well as of certain chemokines, such as MCP-1, involved in monocyte migration. Reduced proliferation of macrophages in response to factors such as macrophage colony-stimulating factor or death of macrophages, including apoptotic attrition, may also contribute to reduced macrophage numbers during lipid lowering. The links between these various pathways and lipid lowering and their relative contributions to the decrease in macrophage number will require further investigation.

Schecter et al recently demonstrated that PDGF induces TF expression in SMCs. We have documented reduced PDGF-B-chain expression during lipid lowering in these same rabbits. Thus, decreased expression of PDGF may limit TF expression by SMCs. We also have reported that lipid lowering promotes accumulation of mature SMCs expressing SMC-specific myosin heavy chain isoforms. An increase in number of SMCs with more normal phenotype by lipid lowering could also contribute to reduced expression of TF.

We recently reported that dietary lipid lowering reduces matrix-degrading enzymes expressed by macrophages and SMCs and promotes interstitial collagen accumulation in these rabbit atheroma, suggesting potential mechanisms of mechanical plaque stabilization. The present study addressed the other critical factor in the potential of a plaque to
precipitate acute clinical consequences, namely its thrombogenicity. The present results suggest that dietary lipid lowering may limit coronary events not only by mechanically stabilizing vulnerable plaques but also by reducing their procoagulant capacity. Some evidence suggests that the clinical benefits of HMG-CoA reductase inhibitors may result from direct effects on lesional cells independent of reductions in LDL. Indeed, a recent report has shown reduced TF expression in isolated monocylic cells exposed to relatively high concentrations of HMG-CoA reductase inhibitors in vitro. Further in vivo experiments should address whether HMG-CoA reductase inhibitors have similar effects in vivo at doses relevant to clinical practice.

### TF Activity

<table>
<thead>
<tr>
<th>Group</th>
<th>TF Activity (mU/100 μg recombinant human tissue factor protein)</th>
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<tbody>
<tr>
<td>Baseline</td>
<td>9.79±2.33</td>
</tr>
<tr>
<td>High</td>
<td>11.10±5.66</td>
</tr>
<tr>
<td>Low</td>
<td>6.13±1.99*†</td>
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</tbody>
</table>

TF activity with a 30-second clotting time was defined as 1 U/mL, and arterial TF activity is expressed as mU/100 μg recombinant human tissue factor protein. Values are mean±SEM.

*P<0.01 vs Baseline group; †P<0.05 vs High group.

In conclusion, the present observations shed new light on the mechanisms by which lipid lowering may reduce clinical complications of atherosclerosis. This study supports the view that lipoproteins or their derivatives can promote local inflammation and thrombogenicity in the arterial wall and that lipid lowering in this context actually constitutes a form of anti-inflammatory and antithrombotic therapy. Our results provide an intellectual framework for understanding biological mechanisms underlying the clinical benefits of lipid lowering. By furnishing direct experimental evidence for
reversibility of inflammatory and thrombogenic stimuli within atheroma, our findings should provide further impetus to treat hyperlipoproteinemia aggressively.

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References


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