Smoking Increases Tissue Factor Expression in Atherosclerotic Plaques
Implications for Plaque Thrombogenicity

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Background—Smoking increases the risk of atherothrombotic events. To determine whether smoking influences plaque thrombogenicity, we examined the effect of cigarette smoking and aspirin use on tissue factor (TF) expression in atherosclerotic plaques.

Methods and Results—A total of 23 apoE−/− mice were exposed to cigarette smoke with (n=9) or without (n=14) aspirin treatment. Eleven mice who were exposed to filtered room air served as controls. Aortic root plaques of mice exposed to smoke had higher immunoreactivity for TF (14±4% versus 6.4±3%; P=0.0005), vascular cell adhesion molecule-1 (15±4% versus 5±2%; P=0.002), and macrophages (16±5% versus 6±2%; P=0.002) compared with nonsmoking controls. Aspirin treatment attenuated smoking-induced changes in plaque composition. In human plaques obtained by carotid endarterectomy, TF immunoreactivity (8±5% versus 2±2%; P=0.0002) and activity (P=0.03) were higher in the plaques from smokers (n=28) than those from nonsmokers (n=28). Aspirin use was associated with reduced TF expression in smokers (9±8% versus 3±4%; P=0.0017).

Conclusions—Our results suggest increased plaque TF expression and thrombogenicity as a novel mechanism for the increased risk of atherothrombotic events in smokers. Treatment with aspirin may reduce TF expression.

Key Words: smoking ■ thromboplastin ■ aspirin ■ atherosclerosis

Smoking doubles the risk of myocardial infarction and ischemic stroke.1 The mechanisms by which smoking increases the risk of these atherothrombotic events are not fully understood. Cigarette smoking confers a hypercoagulable state,2,3 but its effect on plaque thrombogenicity is unknown. Tissue factor (TF) plays a key role in thrombus formation after plaque disruption.4 TF forms a high-affinity complex with factors VII and VIIa; this leads to thrombin formation, which in turn activates the clotting cascade and platelets.5 Smoking increases the number of tissue macrophages,6 which are a predominant source of TF in atherosclerotic plaques. We tested the hypothesis that smoking increases plaque TF expression. We further hypothesized that aspirin, an inhibitor of lipopolysaccharide-induced TF upregulation,7 would attenuate smoking-induced TF upregulation.

Methods

ApoE−/− Mice
Six-week-old male apoE−/− mice were fed high cholesterol chow throughout the study period. At 20 weeks of age, 14 mice (smokers) were exposed to the smoke of half of a research, nonfiltered cigarette each day, 5 days a week, for 8 weeks in a specially designed apparatus, which has been described previously.8 An additional 9 mice who were equally exposed to cigarette smoke were treated with aspirin (0.5 mg/kg SC per day). Blood salicylate levels (determined in 3 mice) were maximal (16.8±2 mg/dL) at 60 minutes after injection. The control group consisted of 11 mice (nonsmokers) exposed to filtered air in a similar apparatus. The study was approved by the Institutional Animal Care and Use Committee.

Immunohistochemistry
After euthanasia at 28 weeks of age, the base of the heart and the first 3 mm of the ascending aorta were embedded in OCT (Tissue Tek, Allegiance). In each mouse, 3 nonconsecutive, 10-μm-thick sections of the aortic root were stained for TF (polyclonal sheep anti-rabbit tissue factor, American Diagnostica). Sections from 5 mice in each group were stained for the expression of vascular adhesion molecule (VCAM-1; rat anti-mouse CD 106, PharMingen) and monocye/macrophages (rat anti-mouse monocyte/macrophages, Serotec).

Western Blot
A total of 50 μg of protein from the aortas of 5 mice in each group were loaded on 12% SDS-PAGE gels, incubated with the anti-TF antibody and then the HRP-conjugated anti-sheep antibody, and detected according to the enhanced chemiluminescence protocol (Amersham).

Aortic TF Expression and Activity
TF procoagulant activity was measured by 1-stage recalcification clotting time, as described previously.9 A total of 25 μL of mouse plasma (Sigma) was mixed with 25 μL of the aortic extracts and 25 μL of 25 mmol/L calcium chloride. Clotting time was measured by

602
a clot timer. For each experiment, a standard curve with lipidated recombinant TF (American Diagnostica) was constructed.

**Patient Population**
A total of 166 consecutive patients underwent carotid endarterectomy for symptomatic carotid disease (cerebrovascular accident, transitory ischemic event, or amaurosis fugax) and 74 (44%) for severe (>80%) asymptomatic carotid artery stenosis.

Twenty-eight patients (17%) were current smokers, 87 (52%) were former smokers, and 51 (31%) had never smoked (nonsmokers). The plaques of 28 smokers and 28 nonsmokers matched for age, sex, and indications for the carotid endarterectomy were studied for TF expression (n=23 in each group) or activity (n=5 in each group).

**TF Expression and Activity in Carotid Plaques**
TF expression was determined using computer-assisted morphometry (Optica 5.1 bioscan) of immunohistochemical staining with murine anti-human monoclonal antibodies against TF (American Diagnostica) and was expressed as percent of plaque area.

Frozen carotid plaque samples were homogenized using CAPS buffer. Protein concentration was determined using the Coomassie blue protein assay (Pierce). TF activity was determined using a commercially available kit (Actichrom TF activity assay, American Diagnostica), which detected TF based on its ability to form TF/VII complex and to cleave Sertozyme-factor VII, resulting in the release of the pNA chromophore. TF activity is presented as lipidized TF concentration (nmol/L) per 100 μg of protein.

**Statistical Analysis**
Normally distributed continuous variables are presented as mean±SD and compared by t test. Otherwise, the variables are compared by Wilcoxon test. Parametric variables are presented as percentage and compared using a 2-tailed χ² or Fisher exact test.

**Results**

**Apoe−/− Mice**
The body weight (33±6, 32±4, and 30±3 g; P=0.3) and cholesterol levels (1383±245, 1419±197, and 1339±306 g/dL; P=0.8) were similar in the smoker, nonsmoker, and smoker mice treated with aspirin, respectively.

**TF Expression**
Plaques from untreated smoker mice had a significantly larger TF immunoreactive area compared with plaques from nonsmoker mice (14±4% versus 6.4±3%; P=0.0005) and smoker mice treated with aspirin (14±4% versus 6.5±4.5%; P=0.002; Figures 1A through 1C). TF was largely located in the lipid-rich core and in the shoulders of the plaques.

By Western blotting, smokers had a 2.3±0.7-fold greater TF content compared with nonsmoker mice (P=0.004). Aspirin treatment reduced TF content to only 1.3±0.17-fold of that of nonsmoker mice (P=0.07; Figure 1D).

Smoker mice also had a larger VCAM-1 immunoreactive area compared with nonsmoker mice (15±4% versus 5±2%; P=0.002) and smoker mice treated with aspirin (15±4% versus 5±3%; P=0.02). Increased VCAM-1 expression was associated with greater macrophage immunoreactivity in smoker compared with nonsmoker mice (16±5% versus 6±2%; P=0.002) and with smoker mice treated with aspirin (16±5% versus 7±5%; P=0.002).

**Aortic TF Procoagulant Activity**
Exposure to smoke was associated with a 2- to 3-fold increase in TF procoagulant activity compared with nonsmoker mice (1.96±1.4 compared with 0.75±0.7 nmol/L TF per 100 μg of protein; n=3 in each group).

**Human Carotid Plaque**

**TF Expression**
Carotid plaques obtained from patients undergoing carotid endarterectomy for symptomatic carotid disease had significantly higher TF immunoreactivity compared with plaques from asymptomatic patients (7.4±7% versus 2.2±3%; P<0.04).

Smokers (n=23) and the matched nonsmokers (n=23) had similar clinical characteristics (Table). TF immunoreactivity was detected in 22 of the 23 plaques from smokers (96%) compared with 14 of the 23 plaques from nonsmokers (61%; P=0.0098). TF immunoreactive area was significantly larger in plaques from smokers compared with those from nonsmokers (8±6% versus 2.2±2%; P=0.0002; Figure 2A). TF colocalized with macrophage immunoreactivity (Figure 2B).

**TF Activity**
TF activity was significantly higher in the plaques from smokers (n=5) compared with those from nonsmokers (n=5; 14.5±2.3 versus 7±2.6 nmol/L lipidated TF per 100 μg of protein; P=0.03).

**Aspirin and TF Expression**
A total of 29 of the patients (63%) were using aspirin (average dose, 208 mg/d) before surgery. The prevalence of smoking among patients treated and untreated with aspirin was similar (48% versus 53%). Aspirin use was associated with a reduced TF-stained area compared with no aspirin treatment (3.6±4% versus 7.5±5%; P=0.0053). Although aspirin use was associated with a significant reduction in TF among smokers (14.5±9% versus 4.4±4%; P=0.0017), the reduction among nonsmokers was not statistically significant (3.4±2% versus 2.0±2%; P=0.4).
Increased circulating thrombin generation and activity, 3 fibrinogen plaques, with attenuation of this effect by prior aspirin use. Increased TF immunoreactivity and activity in human carotid plaques, with attenuation of these effects and (2) smoking is associated with atherosclerotic vascular disease. The clinical significance of these findings is supported by the finding that plaques from patients with asymptomatic severe carotid artery stenosis had lower TF expression compared with those with symptomatic disease, which is similar to findings in coronary atherectomy samples from patients with unstable and stable angina.10

In apoE−/− mice, TF content paralleled VCAM-1 and macrophage immunoreactivity. These findings suggest that smoking may increase plaque macrophage content through increased VCAM-1 expression. A similar mechanism was described in increased monocyte adhesion to endothelial cells exposed to cigarette smoke condensate.11 Whether smoking increases TF expression by enhanced macrophage recruitment alone or if it also upregulates macrophage TF gene expression requires further assessment. Aortic TF procoagulant activity was also higher (2-fold) in mice exposed to smoke. When compared with human plaques, TF activity in mouse aortas was several-fold lower. At least in part, this difference can be explained by the dilution of aortic plaque TF procoagulant activity, because only 15% to 20% of the total aortic surface was covered by atheromas.

The effect of aspirin on plaque TF expression observed in our study is similar to that of cholesterol lowering reported in cholesterol-fed rabbits.13 Cholesterol lowering was implied as a potential mechanism for the powerful preventive effect of statins in patients surviving acute myocardial infarction. Our analogous findings suggest an interesting implication for the beneficial effect of aspirin in patients with occlusive arterial disease.

We conclude that exposure to cigarette smoke is associated with a significant increase in atherosclerotic plaque TF expression and activity, which may in part explain the increased atherothrombotic risk associated with smoking.

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**References**


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