Upregulation of the CD40/CD40 Ligand Dyad and Platelet-Monocyte Aggregation in Cigarette Smokers

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Background—Smoking is a potent cardiovascular risk factor and is associated with proinflammatory and prothrombotic responses. The CD40/CD40 ligand (CD40L) dyad and platelet-monocyte aggregation mediate a range of proinflammatory and prothrombotic processes thought to be important in atherothrombosis. We investigated whether expression of the CD40/CD40L dyad and platelet-monocyte aggregation are altered in cigarette smokers.

Methods and Results—C-reactive protein (CRP), soluble (s) CD40L, and surface expression of CD40L on platelets and T cells and of CD40 on monocytes and platelet-monocyte aggregates were compared in 25 cigarette smokers and 25 age- and gender-matched nonsmokers. Cigarette smokers had increased serum CRP (2.47±2.60 versus 0.94±0.96 mg/L, P=0.008) and appeared to have elevated plasma sCD40L (0.8±1.09 versus 0.37±0.21 ng/mL, P=0.07) concentrations. Smokers also had increased surface expression of CD40 on monocytes (45.9±7.7% versus 39.9±6.5%, P=0.006), of CD40L on platelets (2.9±1.0% versus 2.3±0.6%, P=0.03), and of platelet-monocyte aggregates (26.6±10.9% versus 19.7±8.6%, P=0.02). Plasma cotinine concentrations correlated with monocyte CD40 expression, platelet CD40L expression, and platelet-monocyte aggregates.

Conclusions—Cigarette smokers have upregulation of the CD40/CD40L dyad and platelet-monocyte aggregation that may account for the atherothrombotic consequences of this major cardiovascular risk factor. (Circulation. 2004;109:1926-1929.)

Key Words: smoking ■ inflammation ■ leukocytes ■ platelets

Inflammation plays a central role in the pathogenesis of atherosclerosis and its complications.1 Although smoking has been associated with increases in several inflammatory markers, the biological mechanisms linking smoking and atherosclerosis are complex and have not been fully elucidated.

CD40 ligand (CD40L) and its receptor CD40 are expressed in human atheroma and on a range of atheroma-associated cells, including endothelial cells, smooth muscle cells, mononuclear phagocytes, and platelets.2 Ligation of CD40 mediates an array of proinflammatory effects, including the expression of cytokines, chemokines, adhesion molecules, matrix metalloproteinases, and growth factors.3 These activities are crucial to the process of atherogenesis and promote plaque instability. Indeed, disruption of CD40/CD40L interactions in hypercholesterolemic mice retards the initiation and progression of atherosclerotic lesions.4

CD40L induces tissue factor expression on endothelial cells and monocytes5 and contains an RGD integrin recognition sequence that allows it to bind to glycoprotein Ibβ/Ila, activate platelets, and stabilize arterial thrombi.6 Thus, CD40L has the potential to mediate both proinflammatory and prothrombotic activities within the vasculature. Consistent with these activities, elevation of soluble CD40L (sCD40L) is associated with an increased risk of subsequent cardiovascular events in both healthy women7 and patients with acute coronary syndromes.8

Circulating activated platelets bind to leukocytes, predominantly monocytes, to form platelet-leukocyte aggregates. Adhesion of activated platelets to monocytes induces nuclear translocation of nuclear factor (NF)-κB and expression of interleukin-1β, interleukin-8, monocyte chemotactic protein-1, Mac-1, and tissue factor.9,10 Furthermore, platelet-monocyte aggregation promotes monocyte adhesion to activated endothelium and atherosclerotic lesion formation in apolipoprotein E deficient mice.11 Thus, not only are platelet-monocyte aggregates a sensitive marker of platelet activation, but platelet-monocyte interactions may contribute to the initiation and progression of atherosclerosis.

To date, no study has assessed the effect of smoking on the CD40/CD40L dyad or platelet-monocyte aggregation. Therefore, the aim of the present study was to determine whether the CD40/CD40L dyad and platelet-monocyte aggregation were modified in cigarette smokers.
Methods

Patients and Controls
Twenty-five healthy cigarette smokers were compared with 25 age- and gender-matched healthy nonsmokers. Subjects taking regular medication or who had taken antplatelet agents within the preceding 2 weeks and those with clinical evidence of atherosclerotic vascular disease, body mass index >30 kg/m², hypertension, diabetes mellitus, hypercholesterolemia, an intercurrent illness likely to be associated with an acute phase inflammatory response, and renal or hepatic insufficiency were excluded. Ethical approval was obtained from the local research ethics committee, and all subjects provided written informed consent.

Blood Collection Protocol
Peripheral venous blood was drawn from an antecubital vein through a 19-gauge needle. Blood collected in additive-free tubes was immersed in melting ice and allowed to clot for 1 hour before serum was obtained by centrifugation (1500g for 10 minutes at 4°C). Plasma was prepared from blood anticoagulated with citrate by centrifugation (1500g for 30 minutes). To minimize ex vivo sCD40L release, blood was centrifuged within 5 minutes of collection and separated immediately. Serum and plasma samples were stored at −80°C until analysis.

Measurement of sCD40L, C-Reactive Protein, and Cotinine
Plasma sCD40L concentrations were determined with a commercially available ELISA (detection limit 12 pg/mL, Bender MedSystems). C-reactive protein (CRP) concentrations were measured with an ultrasensitive latex-enhanced immunoturbidimetric assay (Randox Laboratories). Plasma cotinine concentrations were determined by high-performance liquid chromatography (Advanced Bioanalytical Service Laboratories).

Flow Cytometry
The following directly conjugated monoclonal antibodies were obtained from DakoCytomation: phycoerythrin (PE)-conjugated CD154 (TRAP1, IgG1), PE-conjugated CD14 (Tuk-4, IgG2a), PE-conjugated CD 62P (IE3, IgG2a), and their appropriate isotype controls. FITC-conjugated CD42a (GRP-P, IgG1), FITC-conjugated CD14 (UCHM1, IgG2a), FITC-conjugated CD3 (UCHT1, IgG1), PE-conjugated CD40 (LOB7/6, IgG1), and their appropriate isotype controls were obtained from Serotec. Unconjugated CD40L (m1k3a4, Alexis Biochemicals; 24-31, Ancell) and CD40 (ea5, Alexis Biochemicals) monoclonal antibodies were used in blocking experiments.

All immunolabeling was performed in whole blood anticoagulated with D-phenylalanyl-l-prolyl-l-arginine chloromethylketone (PPACK) within 5 minutes of blood sampling. To evaluate CD40 on monocytes and CD40L on T cells, blood was diluted 1:2 with PBS and incubated with the appropriate antibodies for 30 minutes. Thereafter, samples were fixed and the red cells lysed by the addition of 500 μL of FACS-Lyse solution (Becton Dickinson). Platelet-monocyte aggregates were evaluated as described previously.12 For blocking experiments, samples of whole blood were preincubated with monoclonal antibodies (10 μg/mL). To evaluate CD40L on platelets, blood was diluted 1:10 with PBS and incubated with the appropriate antibodies for 20 minutes before the cells were further diluted 1:30 with 1% paraformaldehyde. Monocytes, T cells, and platelets were identified by gating for CD14+, CD3-, and CD42a-positive cells, respectively. Platelet-monocyte aggregates were defined as monocyes positive for CD42a. At least 3000 cells were measured by flow cytometry (Beckman-Coulter Epics XL2). All results are expressed as percentage of positive cells. Analyses were performed with EXPO 32 software (Cytometry Systems).

Statistical Analysis
Continuous variables are reported as mean±SD. Statistical analyses were performed with GraphPad Prism (Graph Pad Software) with Student’s t test, Wilcoxon-Mann-Whitney U test, and Pearson’s and Spearman rank correlation coefficients where appropriate. Statistical significance was taken at P<0.05.

Results
Baseline characteristics were similar (Table), although serum HDL cholesterol concentrations were mildly decreased in smokers (P=0.02).

Effect of Smoking on Serum CRP and Plasma CD40L Concentrations
Serum CRP concentrations (2.47±2.60 versus 0.94±0.96 mg/L, P=0.008) were increased in smokers compared with nonsmokers. Plasma sCD40L concentrations appeared to be higher in smokers, but this was not statistically significant (0.8±1.09 versus 0.37±0.21 ng/mL, P=0.07; Figure 1A).

Effect of Smoking on Cell-Surface Expression of CD40 and CD40L
Cigarette smokers had increased surface expression of CD40 on monocytes (45.9±7.7% versus 39.9±6.5%, P=0.006; Figure 1B) and of CD40L on platelets (2.9±1.2% versus 2.3±0.6%, P=0.03; Figure 1C) compared with nonsmokers. In contrast, there were no differences in the surface expression of CD40L on T cells (35.1±6.0% versus 32.9±4.2%, P=0.14). Recent nicotine intake as measured by plasma cotinine concentrations showed a positive correlation with monocyte CD40 expression (r=0.43, P=0.034; Figure 2A) and CD40L surface expression on platelets (r=0.43, P=0.036; Figure 2B). Similar correlations were found between the number of cigarettes smoked daily and platelet surface expression of CD40L (r=0.42, P=0.037) and surface expression of CD40 on monocytes (r=0.36, P=0.076).

Effect of Smoking on Platelet-Monocyte Aggregates
Platelet monocyte aggregates were increased in subjects who smoked compared with nonsmoking controls (26.6±10.9% versus 19.7±8.6%, P=0.02; Figure 1D). This upregulation of platelet-monocyte aggregation in smokers correlated with plasma cotinine concentrations (r=0.38, P=0.05) and the number of cigarettes smoked daily (r=0.43, P=0.04). In addition, platelet-monocyte aggregates correlated with CD40 expression on monocytes (r=0.41, P=0.046) but not with platelet surface expression of CD40L (r=0.23, P=0.27). Compared with controls, neither CD40 nor CD40L blockade altered platelet-monocyte aggregation (data not shown).

<table>
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<th>Subject Characteristics</th>
<th>Controls</th>
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<tr>
<td>Age, y</td>
<td>35±8</td>
<td>35±8</td>
<td>NS</td>
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<tr>
<td>Male gender</td>
<td>15</td>
<td>15</td>
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</tr>
<tr>
<td>Body mass index, kg/m²</td>
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<td>25.6±3.7</td>
<td>NS</td>
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<td>Total cholesterol, mmol/L</td>
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<td>4.9±1.0</td>
<td>NS</td>
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<tr>
<td>LDL cholesterol, mmol/L</td>
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<td>3.1±0.8</td>
<td>NS</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
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<td>Cigarettes/d</td>
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<td>17±9</td>
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<td>Plasma cotinine, ng/mL</td>
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<td>243.8±111.7</td>
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Discussion
The present study is the first to demonstrate that cigarette smoking is associated with upregulation of the CD40/CD40L dyad and platelet-monocyte aggregation. In addition, we have demonstrated that surface expression of CD40 on monocytes and of CD40L on platelets correlates with recent intake of nicotine. These findings provide an important link between smoking and the development of atherothrombosis.

Although previous studies have demonstrated that smoking is associated with elevation of a number of markers of inflammation, no study has previously assessed the effect of smoking on the CD40/CD40L dyad. The CD40/CD40L dyad is of particular interest because of its distribution and multifunctionality. The present study demonstrated that smoking is associated with increased expression of the CD40/CD40L dyad. Consistent with the direct relationship between cardiovascular risk and cigarette consumption, surface expression of both CD40 on monocytes and CD40L on platelets was positively correlated with plasma cotinine concentrations and the number of cigarettes smoked. Recent studies have demonstrated that diabetes mellitus and hypercholesterolemia are also associated with upregulation of the CD40/CD40L dyad. Thus, it appears the CD40/CD40L dyad may be an important mechanism linking a number of the traditional cardiovascular risk factors to the development of atherothrombotic events.

We have demonstrated that cigarette smoking is associated with increased platelet-monocyte aggregation. Platelet-monocyte aggregation is not only a sensitive measure of platelet activation but also has important proinflammatory consequences. In the present study, platelet-monocyte aggregates correlated positively with CD40 expression on monocytes. Because platelet-monocyte adhesion is known to activate NF-κB, and this transcriptional activator is thought to be important in the regulation of CD40 gene expression, we hypothesize that platelet-monocyte adhesion may also induce the expression of CD40 on monocytes. Moreover, by bringing activated platelets that express CD40L into contact with monocyes that express CD40, platelet-monocyte aggregation will facilitate CD40 ligation. Thus, increased platelet-monocyte interactions will have a number of proinflammatory and prothrombotic effects that may contribute to the pathogenesis of atherosclerosis.

Neither CD40 nor CD40L blockade had an effect on platelet-monocyte aggregation, which excludes a direct causal relationship between these 2 phenomena. This is consistent with our previous findings that platelets bind to monocyes primarily via P-selectin-P-selectin glycoprotein ligand-1 interactions.

Study Limitations
We have studied the effect of chronic smoking in a selected healthy population at a single time point. As such, this study cannot establish a causal relationship between cigarette smoking and increased levels of CD40/CD40L and platelet-monocyte aggregation.
The populations were matched for age and gender, and subjects with other risk factors for cardiovascular disease were excluded to minimize possible confounding factors. The only difference in baseline characteristics was the well-described reduction of serum HDL cholesterol concentrations seen in cigarette smokers.\textsuperscript{15}

**Conclusion**

In conclusion, we have demonstrated that cigarette smoking is associated with upregulation of the CD40/CD40L dyad and platelet-monocyte aggregates. We propose that these effects may provide a major contribution to the mechanisms whereby cigarette smoking promotes atherogenesis and is associated with the development of adverse cardiovascular events.

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**References**

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