Hyperfibrinogenemia Is Associated With Specific Histocytological Composition and Complications of Atherosclerotic Carotid Plaques in Patients Affected by Transient Ischemic Attacks

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Background—Epidemiological studies have demonstrated that hyperfibrinogenemia is an independent risk factor for cerebrovascular atherosclerosis. However, the underlying mechanisms are poorly understood. We studied whether hyperfibrinogenemia could modify the histological composition of atherosclerotic plaque and precipitate carotid thrombosis resulting from rupture of the plaque.

Methods and Results—We studied the histological composition of 71 carotid atherosclerotic plaques from patients who had undergone surgical endarterectomy after a first episode of transient ischemic attack. Patients were divided into 3 groups corresponding to the tertiles of plasma fibrinogen values. Hypercholesterolemia, hypertriglyceridemia, hypertension, diabetes, and smoking habit were also assessed. At the histological analysis, plaques of patients in the highest tertile of fibrinogen (>407 mg/dL) were characterized by a high incidence of thrombosis (66.7% of cases) compared with plaques of subjects in the lower (21.7%) (P=0.002) and middle (29.2%) (P=0.009) tertiles. Plaque rupture was significantly associated with high fibrinogen levels (54.2%, P=0.003). Multivariate logistic regression indicated that hyperfibrinogenemia was an independent risk factor for a decrease in cap thickness (P=0.0005), macrophage foam cell infiltration of the cap (P=0.003), and thrombosis (P=0.003). When the presence of other risk factors was accounted for, hyperfibrinogenemia remained an independent predictor of carotid thrombosis with an odds ratio of 5.83, compared with other risk factors.

Conclusions—The results of the present study add to the evidence that hyperfibrinogenemia, independently of other risk factors, is associated with a specific histological composition of carotid atherosclerotic plaques that predisposes them to rupture and thrombosis. (Circulation. 2000;101:744-750.)

Key Words: fibrinogen ■ atherosclerosis ■ carotid arteries ■ histology ■ thrombosis

Histopathological studies have revealed that human atherosclerotic plaques are characterized by heterogeneous composition. In carotid arteries, the histological composition of the plaque seems to be an important factor for the onset of cerebrovascular accidents, especially in relation to the embolic properties of complicated plaques.

Although age, male sex, hypercholesterolemia, hypertension, smoking, and diabetes correlate with plaque burden found at autopsy, only few studies have correlated the presence of these risk factors with the histological characteristics of atherosclerotic plaques. Likewise, risk factors may influence the histomorphological composition of the plaque and trigger particular complications, such as hemorrhage, rupture, and thrombosis, and consequently the onset of clinical symptoms.

Both case-control and prospective cohort studies have consistently shown that hyperfibrinogenemia is positively related to coronary and cerebrovascular diseases. In addition, clinical studies have shown that patients with progressive carotid atherosclerosis have significantly higher fibrinogen levels than those with nonprogressing lesions.

Despite the evidence linking increased plasma fibrinogen levels to acute cerebrovascular events, little is known about the possible association between hyperfibrinogenemia and the histological composition of carotid atherosclerotic plaques.
Therefore, we designed this study to test the hypothesis that in patients affected by transient ischemic attacks (TIAs), hyperfibrinogenemia is associated with a specific histomorphological composition of carotid atherosclerotic plaques and that the presence of this risk factor precipitates carotid thrombosis resulting from plaque rupture.

Methods

Selection of Cases
From January 1994 to December 1996, patients who suffered a first episode of TIA and underwent carotid endarterectomy (CE) at the University of Rome Tor Vergata formed the basis of the study. All patients were treated with aspirin (125 mg/d) after the hospital admission and were free of inflammatory diseases.

Study Population
Carotid plaques from 71 patients (60 men and 11 women, mean age 64.2±7.64, range 44 to 82 years) were studied after surgical CE. The interval between the onset of clinical symptoms and surgery was 15±8 days. All patients showed a carotid stenosis >50% at the angiographic and ultrasonographic examination. The presence of cerebral lesions was excluded by a CT scan study.

Determination of Plasma Fibrinogen
Determination of plasma fibrinogen was performed before surgery and 12 months after the CE according to the method of Wilhelmsen and coauthors. All tests were carried out immediately and performed in triplicate, with a minimum interval of 3 days between each measurement. The assay was performed with an automated analyzer (ACL analyzer, Instrumentation Laboratory Co) and a PT-fibrinogen HS kit as recommended by the manufacturer (Instrumentation Laboratory). Because a uniform cutoff value of plasma fibrinogen for the diagnosis of hyperfibrinogenemia has not been defined, we divided the patients into 3 groups according to tertiles of plasma fibrinogen values.

Assessment of Risk Factors
To rule out the influence of risk factors other than hyperfibrinogenemia on plaque histomorphological composition, the patient’s risk factor profile was also assessed. Hypertension was determined by a case history of antihypertensive drug treatment. Patients either affected by insulin-dependent diabetes or treated with diet and/or oral hypoglycemic agents were included in the diabetic group. A smoking habit of ≥20 cigarettes/d over the previous 2 years classified patients as current smokers. Patients either with total serum cholesterol >210 mg/dL or treated in the previous 12 months with a lipid-lowering drug were considered hypercholesterolemic. Finally, hypertriglyceridemia was documented either on the basis of the case history or when the serum triglyceride value exceeded 200 mg/dL.

Histological Sampling and Light Microscopy
At surgery, the carotid plaques were removed en bloc by a careful operative technique that preserved the plaque structure. The samples were fixed in 10% buffered formalin for 24 hours immediately upon removal. According to the sampling method previously reported, the specimens, after decalcification, were sliced transversely every 3 mm. Each carotid slice was removed and numbered to allow the sequential reconstruction of the entire plaque length. On the whole, 3 to 10 slices of each plaque were sampled, according to its size, embedded in paraffin, and stained with hematoxylin-eosin, Weigert-van Gieson, Alcian blue–PAS, and Movat pentachrome.

Thus, the entire plaque was evaluated for the presence of a necrotic core, calcification, intraplaque hemorrhage, and thrombosis associated with plaque rupture or erosion. Arterial segments showing thrombosis or, in the absence of an acute thrombus, those with the most severe degree of stenosis were chosen for the immunohistochemical and morphometric analysis. Two pathologists (A.M., G.P.) who were blinded to the clinical findings graded all histocytological components of the plaques. Intraobserver variability was 95%.

Thrombosis was defined according to Carr et al and divided into 2 categories: thrombosis associated with plaque rupture or with plaque erosion.

Immunohistochemical Studies
The immunohistochemical study was used to characterize and quantify the cell types present in the plaque. Serial paraffin sections 5 μm thick were incubated with the following primary monoclonal antibodies: α-smooth muscle actin, CD68 (anti-human macrophage; Dakopatts), CD3 (anti-human T cell; Dakopatts), and CD20 (anti-human B cells; Dakopatts). Cell counting was done at a magnification of ×400 with a test grid with an area of 0.22 mm². We counted either an average of 20 fields per section or a number of microscopic fields until the SEM was <5%.

Statistical Analysis
Data were analyzed by SPSS (Statistical Package for the Social Sciences) software. Patients were grouped into tertiles according to values of plasma fibrinogen. Pearson’s χ² test was used to assess possible differences for sex, hypertension, diabetes, and smoking between patients in the lower, middle, and highest tertiles of fibrinogen values. The effects of age, serum cholesterol, and triglycerides were assessed by 1-way ANOVA. Pearson’s χ² test was also used to assess differences between the 3 groups of fibrinogen values for the presence of single histocytological components of the plaque. For this analysis, continuous numerical variables (minimum thickness of the fibrous cap, number of macrophagic foam cells, and lymphocytes) were divided into 2 groups, with the value corresponding to the 50th percentile used as cutoff. Differences in fibrinogen plasma values at the time of enrollment and after 12 months were assessed by paired t test.

Multivariate analysis using logistic regression (significance level for removing=0.4, for entering=0.2) was used to identify (1) a subset of histocytological components of the carotid plaque, which correlates significantly with highest levels of fibrinogen, and (2) independent risk factors, which significantly correlate with thrombosis, plaque rupture, macrophage infiltration, and thinning of the cap.

The possible effect of interaction of the different risk factors on thrombosis, plaque rupture, macrophage infiltration, and cap thinning was calculated by log-linear analysis.

Values are given as percentage or as mean±SEM. In all analyses, a value of P<0.05 was considered statistically significant.

Results

Association Between Risk Factors
Fibrinogen plasma values at the time of enrollment ranged from 190 to 577 mg/dL, with a mean of 366.9±11.4 mg/dL. Independently of fibrinogen values, 13 subjects showed a single risk factor; 24 patients, 2 risk factors; 21 patients, 3 risk factors; 7 patients, 4 risk factors; and 2 patients, 5 risk factors.

The differences we observed in risk factor distribution among groups with different tertiles of fibrinogen were not statistically significant. No differences were even detected in the same patient between fibrinogenemia detected at the time of enrollment and that measured 12 months after the CE. In particular, the mean fibrinogen value of subjects in the highest tertile was 463.75±34.95 mg/dL at the time of enrollment and 492.87±61 mg/dL after 12 months (P=0.67).

Hyperfibrinogenemia and Plaque Composition
Immunohistochemical study demonstrated that plaques from patients affected by hyperfibrinogenemia were characterized by the presence of a high number of inflammatory cells composed of monocytes/macrophages, CD68-positive cells...
(Figure, B), and T lymphocytes (CD3-positive) localized mainly in the shoulder and in the cap of the plaque (Figure, D). A small number of B lymphocytes (L26-positive) were also observed. As shown by the quantitative analysis, a significantly higher number of macrophage foam cells characterizes the plaque cap of patients in the highest tertile compared with those belonging to the middle \( (P=0.009) \) and lower \( (P<0.001) \) tertiles (Table 1). Smooth muscle cells (\( \alpha \)-smooth muscle actin-positive) were scant and consisted of elongated cells with fusiform nuclei and a cytoplasm often totally occupied by lipid droplets (Figure C).

Moreover, a significant inverse relationship between plasma levels of fibrinogen and thickness of fibrous cap was observed in the plaques of our patients. A fibrous cap with a minimum thickness <210 \( \mu \)m occurred more frequently in plaques of patients of the highest tertile (83.3\% of cases, 20 of 24) than in those of the middle \( (P=0.001) \) and lower tertiles \( (P<0.001) \) (Table 1).

Hyperfibrinogenemia, Plaque Rupture, and Thrombosis

Thrombosis (Figure, A) was observed in 66.7\% of the patients belonging to the highest tertile (16 of 24 plaques) and was significantly more frequent than in the plaques from subjects in the lower and middle tertiles (21.7\% of cases, \( P=0.002 \), and 29.2\%, \( P=0.009 \), respectively) (Table 1).

In patients in the highest tertile of fibrinogen (13 versus 3 cases), thrombosis was associated mainly with plaque rupture rather than erosion. Accordingly, the occurrence of rupture was significantly greater in plaques of this group of patients than in those in the lower \( (P=0.003) \) and middle \( (P=0.02) \) tertiles (Table 1).

Finally, multivariate logistic regression indicated that the presence of thrombosis \( (P=0.03) \), minimum cap thickness <210 \( \mu \)m \( (P=0.02) \), and macrophage foam cell infiltration of the cap \( (P=0.03) \) were the morphological characteristics more strongly correlated with the presence of high fibrinogen levels.

Relation Between Risk Factors, Thrombosis, and Plaque Rupture

Multivariate logistic regression showed that values of fibrinogen >407 mg/dL and hypercholesterolemia were independent predictors of carotid thrombosis and plaque rupture (Table 2). However, log-linear analysis demonstrated no statistically significant interaction between the highest fibrinogen values and hypercholesterolemia on the presence of carotid thrombosis \( (P=0.78) \) and plaque rupture \( (P=0.24) \).

Similarly, no significant interaction was evident between hyperfibrinogenemia (highest tertile) and hypertension on the presence of high numbers (>23 cells/mm\(^2\)) of macrophage foam cells within the fibrous cap.
Finally, an increased risk of thrombosis characterized the plaques of patients in the highest tertile of fibrinogen (odds ratio, 5.83) compared with that observed either in patients included in the middle tertile (odds ratio, 3.31) or in those affected by hypercholesterolemia (odds ratio, 2.93) (Table 2). Likewise, plaques of patients in the highest tertile of fibrinogen showed the highest odds ratio for cap thinning, macrophage infiltration of the cap, and plaque rupture compared with patients with other risk factors (Tables 2 and 3).

**Discussion**

In the present study, we evaluated the effect of hyperfibrinogenemia on the histomorphological composition of the carotid plaque in patients affected by TIA and undergoing surgical CE. Our findings suggest that hyperfibrinogenemia is associated with a particular histological composition of carotid plaques, which in turn may predispose to plaque rupture and thrombosis.

Clinical studies have demonstrated the benefit of CE in patients with symptomatic cerebrovascular disease. In particular, the results of the European Carotid Surgery Trial (ECST) and the North American Symptomatic Carotid Endarterectomy Trial (NASCET) suggest that surgery is indicated when the degree of stenosis is >75%. However, studies on the natural history of asymptomatic carotid plaques demonstrated that in addition to the grade

**TABLE 1.** Histological Characteristics of Plaques of Patients With the Various Tertiles of Fibrinogen (Univariate Analysis)

<table>
<thead>
<tr>
<th>Tertiles of Plasma Fibrinogen</th>
<th>Minimum cap thickness</th>
<th>Cap macrophagic foam cells</th>
<th>Cap T lymphocytes</th>
<th>Cap smooth muscle cells</th>
<th>Necrotic lipidic core</th>
<th>Intimal vascularization</th>
<th>Calcification</th>
</tr>
</thead>
<tbody>
<tr>
<td>I, 190–330 mg/dL (n = 23)</td>
<td>6 (26.1)</td>
<td>6 (23.9)</td>
<td>16 (69.6)</td>
<td>11 (47.8)</td>
<td>14 (60.9)</td>
<td>20 (87.0)</td>
<td>18 (78.3)</td>
</tr>
<tr>
<td>II, 331–406 mg/dL (n = 24)</td>
<td>9 (37.5)</td>
<td>15 (62.5)</td>
<td>15 (62.5)</td>
<td>13 (54.2)</td>
<td>10 (41.7)</td>
<td>16 (66.7)</td>
<td>16 (66.7)</td>
</tr>
<tr>
<td>III, 407–577 mg/dL (n = 24)</td>
<td>20 (83.3)</td>
<td>4 (16.7)</td>
<td>6 (25.0)</td>
<td>14 (58.3)</td>
<td>12 (50.0)</td>
<td>17 (70.8)</td>
<td>17 (70.8)</td>
</tr>
<tr>
<td>P</td>
<td>0.40</td>
<td>0.01</td>
<td>0.001</td>
<td>0.40</td>
<td>0.66</td>
<td>0.37</td>
<td>0.37</td>
</tr>
</tbody>
</table>

**TABLE 2.** Risk Factors and the Presence of Thrombosis and Rupture of the Carotid Plaques

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Thrombosis</th>
<th>P</th>
<th>Odds Ratio</th>
<th>P</th>
<th>Odds Ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma fibrinogen &gt;407 mg% (highest tertile) (n = 24)</td>
<td>16 (66.7)</td>
<td>0.0008</td>
<td>5.83 (1.99–17.05)</td>
<td>0.001</td>
<td>5.76 (1.91–17.41)</td>
<td></td>
</tr>
<tr>
<td>Hypercholesterolemia (n = 37)</td>
<td>19 (51.4)</td>
<td>0.03</td>
<td>2.93 (1.08–7.95)</td>
<td>0.008</td>
<td>4.42 (1.40–13.96)</td>
<td></td>
</tr>
<tr>
<td>Hypertension (n = 31)</td>
<td>7 (36.8)</td>
<td>0.79</td>
<td>0.86 (0.29–2.55)</td>
<td>0.82</td>
<td>1.14 (0.36–3.55)</td>
<td></td>
</tr>
<tr>
<td>Diabetes (n = 25)</td>
<td>9 (36.0)</td>
<td>0.66</td>
<td>0.80 (0.29–2.18)</td>
<td>0.83</td>
<td>0.89 (0.30–2.60)</td>
<td></td>
</tr>
<tr>
<td>Smoker (n = 50)</td>
<td>21 (42.0)</td>
<td>0.49</td>
<td>1.45 (0.50–4.21)</td>
<td>0.65</td>
<td>0.77 (0.26–2.33)</td>
<td></td>
</tr>
</tbody>
</table>
of stenosis,27,28 the histomorphological composition of carotid plaques plays a fundamental role in the transformation of a clinically silent lesion into a symptomatic one.4,5 In particular, reviewing the data from patients of the NASCET Trial, Eliasziw and associates found a higher risk for subsequent stroke in patients with angiographic evidence of plaque ulcer.29

It appears now that the debate in atherosclerosis research has indeed moved on from whether to treat to whom to treat. In agreement with this trend, our present study demonstrates for the first time that hyperfibrinogenemia, independently of other risk factors, is associated with macrophage cap infiltration and a decrease in plaque cap thickness, which in turn are associated with carotid plaque rupture and thrombosis. Although the role of plaque rupture, cap thinning, and inflammatory infiltration of the cap in the pathophysiology of cerebrovascular accidents is not clearly defined, plaque rupture and thrombosis nevertheless are acute events and, as demonstrated in acute coronary syndromes, are probably associated with the progression of a mature asymptomatic plaque into a symptomatic lesion.5

The present study confirms this opinion and further strengthens previous observations8–10 that demonstrated that the morphological heterogeneity of the atherosclerotic plaque is not a haphazard phenomenon but instead is significantly correlated with the presence of different risk factors.

Although several epidemiological studies have demonstrated that high levels of fibrinogen correlate with an increased incidence of myocardial infarction, stroke, and TIA,11–13,30 whether hyperfibrinogenemia is a cause or an effect of acute clinical events is still unclear. In particular, it has been demonstrated that plasma fibrinogen increases within hours after a stroke and thereafter gradually falls to normal levels within the next few weeks.31 Therefore, increased fibrinogen levels in patients affected by stroke could be considered the result of an acute-phase reaction subsequent to brain tissue necrosis.32 Conversely, fibrinogen levels, despite being significantly elevated in patients with TIA, do not rise after a transient ischemic episode, which suggests that elevated plasma fibrinogen level may be a causative factor in the onset of this cerebrovascular event.33 Consequently, a related issue is whether elevation of plasma fibrinogen level is an indirect nonspecific marker of inflammation within the atherosclerotic vessel wall or has a direct pathophysiological role in the modification of histological characteristics of carotid plaques that promote rupture.

Several studies have shown that acute-phase reactant proteins, such as fibrinogen and C-reactive protein (CPR), are mainly influenced directly by interleukin-6 and indirectly by interleukin-1 and tumor necrosis factor-α.33,34 Both fibrinogen and CPR are risk indicators for cardiovascular events.35,36 Recently, in the ECAT Angina Pectoris Study, fibrinogen turned out to be an independent predictor of acute clinical events even when CPR was included in a multivariate model. These data suggest that the association of fibrinogen with clinical events cannot be explained solely as a marker of an inflammatory state.37 In our study, if hyperfibrinogenemia had been related to a plaque inflammatory state, we should have seen a significant decrease of fibrinogen level after surgical removal of the plaque. On the contrary, after 1 year of follow-up, no significant modifications of fibrinogen levels were observed in any patient enrolled in this study. Thus, our findings support the opinion37 that hyperfibrinogenemia is not specifically related to a plaque inflammatory state.

### Carotid Plaque Vulnerability and Risk Factors

Recently, Burke and coauthors10 reported that hypercholesterolemia was the most important predictor of plaque rupture and thrombosis in patients who suffered sudden coronary death. In our study, the correlation between the presence of thrombotic plaques and high levels of fibrinogenemia was so strong that the increase of thrombogenic risk in plaques from patients with plasma fibrinogen >407 mg/dL was 5.83 compared with an odds ratio value of 2.93 in patients with hypercholesterolemia (Table 2). This finding may reflect a different mechanism of action of those 2 risk factors in carotid plaques. In fact, we did not find any significant association between hypercholesterolemia and inflammatory infiltration of the cap (Table 3). In addition, the fact that none of the other risk factors evaluated in this study, with the exception of hypercholesterolemia and hyperfibrinogenemia, were significantly associated with thrombosis confirms the hypothesis that other pathogenetic mechanisms should be involved in TIA development. A vasoconstriction related to smoking habit, an intramural hemorrhage, or a release of embolic material due to the presence of the friable necrotic-
l lipid core of a plaque could be such a pathogenetic mechanism.

Study Limitations
The first limitation of the present study is that we examined only patients affected by TIA. Further histological studies in asymptomatic patients or in subjects affected by stroke are needed to better establish the effect of high fibrinogen levels on the histomorphological composition of carotid plaque.

The second limitation is represented by the presence of >1 risk factor in most of the patients examined. Nevertheless, the multivariate statistical model we used has shown an independent role of hyperfibrinogenemia in the development of the specific histomorphological characteristics of the plaque that are responsible for thrombosis. Although other risk factors were present in patients with high levels of fibrinogen, their incidence in the groups belonging to the different tertiles of fibrinogen did not vary significantly. In addition, the logarithmic analysis further confirmed the role of hyperfibrinogenemia in the development of plaque complications. This has been proved by the lack of significant interactions between hyperfibrinogenemia and other risk factors on macrophage plaque infiltration, cap thinning, plaque rupture, and thrombosis.

Conclusions
Data from our patient population support the conclusion that hyperfibrinogenemia, compared with other risk factors, correlates significantly with a particular histological composition and complications of carotid atherosclerotic plaques.

Therefore, our study helped to identify a subset of patients at high risk for a rapid progression of carotid atherosclerosis and related clinical events. We suggest that these patients may benefit from an earlier surgical intervention even if the degree of carotid stenosis is not severe.

Acknowledgment
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