Severity of Peripheral Atherosclerosis Is Associated With Fibrinogen and Degradation of Cross-linked Fibrin

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Immunohistochemical studies of human atherosclerotic lesions have demonstrated the occurrence of fibrin deposition and its degradation in the arterial wall. We studied fibrinogen, the generation of thrombin, and the degradation of fibrin in 40 patients with stable peripheral arterial occlusive disease of varying severity, as assessed by the ankle/brachial pressure index and duplex ultrasonography and/or angiography. Circulating fibrinogen (functional and immunological), fibrinopeptide A, thrombin–antithrombin III complex, and D-dimer were measured. The severity of atherosclerosis was associated with both fibrinogen (both functional and immunological) and D-dimer ($r = .57$, $P < .0002$, and $r = .57$, $P < .0001$, respectively). Fibrinogen and D-dimer showed a significant positive correlation ($r = .50$, $P < .001$).

Generation of thrombin was detected in 24 patients (60%) by fibrinopeptide A and levels of thrombin–antithrombin III complex. As a sign of coagulation activation and fibrinolysis, we found that thrombin–antithrombin III complex and the degradation of cross-linked fibrin were progressively associated with the extent of vascular disease. The plasmin-mediated fibrin breakdown contributed to increased levels of circulating fibrinogen, an established risk factor for thrombotic complications. The significant correlations between fibrinogen/D-dimer and the severity of atherosclerosis support previous pathological studies and imply that local degradation of cross-linked fibrin is involved in the progression of atherosclerosis.

KEY WORDS: fibrinogen • fibrinopeptide A • thrombin–antithrombin III complex • D-dimer • peripheral arterial occlusive disease • atherosclerosis

The interaction between blood and the diseased arterial wall is the underlying mechanism for the progression of cardiovascular diseases. Rupture of an atherosclerotic plaque, with the sudden exposure of subendothelial vessel wall components, is immediately followed by deposition of platelets and activation of coagulation, leading to either clinical occlusive or subclinical mural thrombosis. Recent pathological studies of postmortem arteries and samples obtained during reconstructive vascular surgery relate the progression of atherosclerosis to the extent of fibrin deposition and its degradation products in the arterial wall.4–6 These experimental findings suggest that there is continuous deposition and lysis of fibrin within the arterial wall.4

There has been a lack of ideal clinical markers for monitoring pathophysiological processes involved in cardiovascular diseases.6 Recently some studies have successfully applied certain plasma markers for assessing the activation of coagulation and subsequent fibrinolysis during acute massive thrombosis, such as deep vein thrombosis and pulmonary embolism, as well as disseminated intravascular coagulation.6–9 In acute ischemic stroke, myocardial infarction, or early occlusion after coronary artery angioplasty, arterial thrombosis has been associated with the formation of thrombin and plasmin.10–12 On the other hand, when thrombosis is restricted to the injured site of an artery, such as in unstable angina, the same plasma markers have proved less sensitive.13 Taken together, these data illustrate that the extent of the thrombotic process—whether occlusive and leading to soluble circulating complexes, or locally restricted to the vessel wall injury—modifies the feasibility of different markers. Furthermore, it is unknown to what extent the “background” of thrombosis, ie, atherosclerosis itself, affects these markers.

We wanted to study fibrinogen, the generation of thrombin, and the degradation of fibrin in patients with stable and naturally occurring peripheral arterial occlusive disease (PAOD) of varying severity. We chose patients with PAOD because this disease affects a prominent area of the arterial tree in comparison with patients who have coronary artery disease (CAD) or cerebrovascular disease. The severity of atherosclerosis was assessed functionally by measurement of ankle/brachial pressure indices (ABI) and graded by duplex ultrasonography and/or angiography. We studied the association between the intensity of vascular insufficiency and the circulating levels of fibrinogen, thrombin–antithrombin III (TAT) complex, fibrinopeptide A (FpA), and the plasmin-induced degradation product of cross-linked fibrin, D-dimer.

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Methods

Patients

The vascular laboratory and radiological assessments of PAOD and blood sample collection were performed at the Fourth Department of Surgery, Helsinki University Central Hospital, with the approval of the institutional review board. Informed consent was obtained from all study subjects. Forty consecutive patients with symptomatic PAOD were included in this study. To study the natural occurrence of atherosclerosis, all previous cardiovascular surgery and/or angioplasty procedures were regarded as exclusion criteria. Patients with rest pain or skin lesions were not included. To avoid acute thrombotic events, patients with a sudden worsening of symptoms over the 3 months preceding the evaluation were not included. Two patients were excluded because of a thrombus in an aortic aneurysmal sac as detected by ultrasound and were replaced by other patients for the study.

The mean age of the patients was 64 years (range, 37 to 85 years); 25 were male and 15 were female. Assessment of risk factors for PAOD revealed 33 (82%) smokers/ex-smokers according to self-reported cigarette use, 13 (32%) hypertensives, and 5 (12%) diabetics (1 diabetes type I and 4 type II). The criteria for CAD, found in 11 (28%) patients, were classical symptoms of angina pectoris in everyday life, ischemic electrocardiographic changes during treadmill exercise testing, and/or documented myocardial infarction. Two (5%) patients had a history of transient ischemic attacks and/or stroke, indicating cerebrovascular disease. Eighteen (45%) patients did not take any medication, while 14 (35%) were taking acetylsalicylic acid; 11 (28%), \( \beta \)-blockers; and 5 (12%), nitrates. The patients did not suffer from other systemic diseases.

Assessment of PAOD

PAOD was assessed clinically by a vascular surgeon and objectively by noninvasive blood pressure measurements at rest and by color-coded duplex ultrasonography (ATL Ultramark 9; ATL Inc, Seattle, Wash) and/or angiography.\(^{14} \) Arterial blood pressure was recorded twice by sphygmomanometry with the patient in a supine position. The ratio of ankle to brachial systolic pressures of the posterior tibial and dorsal artery of the foot were recorded by using a hand-held Doppler device as the distal detector (directional Doppler model 806-C; Parks Electronics Lab, Beaverton, Ore). Mean ABI indicates a mean value measured from both extremities, and a worse ABI is represented by a lower index.

Additionally, 36 patients were studied by either duplex ultrasonography and/or angiography of lower-limb arteries. Duplex scanning was performed primarily on patients (n=32) who had originally been scheduled for conservative treatment. Furthermore, angiography of the arteries of the lower extremities was performed on 15 patients who were evaluated for vascular surgery. The number and degree of diametrical stenoses were analyzed within both aortocruial segments. The severity of atherosclerosis was graded according to the tightest stenosis of the lower-limb arterial tree as follows: (1) presence of <50% stenosis, (2) presence of >50% stenosis, (3) presence of total occlusion, and (4) presence of two or more total occlusions.

Blood Sampling

Nonfasting blood samples were taken between 9 and 12 AM. Blood was obtained from patients in a supine position after a 20-minute rest. Blood without stasis was collected via venipuncture with a 17-gauge plastic cannula (polytetrafluoroethylene; Viggo AB, Helsingborg, Sweden).

The first 3 mL was collected in a serum tube for routine measurements of creatinine (kinetic method; Boehringer Mannheim, Mannheim, Germany), total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides (enzymatic methods; Boehringer Mannheim), and C-reactive protein (immunochemical method; Orion Diagnostica, Espoo, Finland). Low-density lipoprotein (LDL) cholesterol concentration was calculated according to the Friedewald formula: total cholesterol minus HDL minus triglycerides divided by 5.\(^{15} \)

Subsequently, blood (9 volumes) for hemostatic and fibrinolytic samples was collected in polypropylene tubes containing sodium citrate (0.11 mol/L) as an anticoagulant (1 volume). The sample for FpA was collected in a special anticoagulant, and fibrinogen was extracted with ethanol before the sample was frozen, as has been previously described.\(^{16} \) For the \( \phi \)-dimer sample, aprotinin (0.2 TIU/mL; Sigma Chemical Co, St Louis, Mo) was added to the anticoagulant. The samples were centrifuged (10 minutes, 1300g, 22°C) without delay, and the plasma was separated into polypropylene tubes to be frozen and stored at −20°C (\( \phi \)-dimer at −70°C).

Analysis of Hemostatic and Fibrinolytic Samples

The samples were analyzed within 3 months. Plasma was briefly thawed at 37°C for processing. Fibrinogen was assessed by both the functional method of Clauss (Baxter Healthcare Corp, Miami, Fla; normal range, 2.0 to 4.0 mg/mL) and an immunonephelometric method (Behring, Marburg, Germany; normal range, 3.0 to 6.0 mg/mL).\(^{17} \) TAT (normal, <3.0 ng/mL), FpA (normal, <3.0 ng/mL), and \( \phi \)-dimer (normal, <400 ng/mL) were analyzed with an enzyme-linked immunosorbent assay (Enzygnost TAT; Behring, Marburg, Germany, and Asserachrom FpA and Asserachrom D-Di; Diagnostica Stago, Asnieres-sur-Seine, France, respectively). Plasma obtained from 20 healthy age- and sex-matched control subjects was analyzed for TAT and \( \phi \)-dimer.

Statistical Analysis

The results are expressed as means plus the range and the 95% confidence intervals (CIs). Regression analysis was applied to study the associations between markers. Factorial ANOVA was used when pertinent.

Results

Although PAOD as assessed by ABI was in accordance with the results obtained by duplex ultrasonography/angiography: the mean ABI was significantly lower, 0.45 (95% CI, 0.31 to 0.60), in patients (n=9) with the severity of grade 4 than in others (n=27) with...
an ABI of 0.67 (95% CI, 0.61 to 0.72). The 11 patients with other manifestations of atherosclerotic disease had a lower mean ABI of 0.57 (95% CI, 0.45 to 0.69) than the 29 patients without signs of coronary or cerebrovascular affection (ABI of 0.64; 95% CI, 0.57 to 0.71). The mean serum creatinine of 87 μmol/L (range, 59 to 131 μmol/L) fell within normal limits. Mean serum lipid values of the patients were acceptable except for triglycerides, which were elevated (2.5 mmol/L; 95% CI, 1.9 to 3.1 mmol/L) among males. C-reactive protein was <10 mg/mL in 34 patients and ranged between 14 and 32 mg/mL in 6 patients.

Mean fibrinogen levels were high, at 4.1 mg/mL (range, 2.6 to 5.9 mg/mL; 95% CI, 3.8 to 4.3 mg/mL), as assessed by the method of Clauss, and 5.1 mg/mL (range, 3.0 to 9.1 mg/mL; 95% CI, 4.6 to 5.5 mg/mL) by the immunonephelometric method. The correlation between the fibrinogen values obtained by the two methods was excellent (r=0.92, P<.0001). All other significant correlations between hemostatic/fibrinolytic parameters and ABI are presented in Fig 1A and the Table. The fibrinogen (functional and immunological) levels were very significantly associated with the severity of the disease as assessed by both the mean ABI and the ABI of the worse leg (Fig 1A and the Table).

In 24 patients, both FpA at 7.3 ng/mL (range, 1.4 to 23.0 ng/mL; 95% CI, 5.3 to 9.3 ng/mL) and TAT at 3.7 ng/mL (range, 2.0 to 8.0 ng/mL; 95% CI, 3.2 to 4.1 ng/mL) were higher than normal. D-dimer levels in plasma, at 665 ng/mL (range, 258 to 1988 ng/mL; 95% CI, 541 to 770 ng/mL), were significantly elevated in patients. Furthermore, the association of d-dimer with ABI was highly significant (Fig 1B and the Table).

The patients (n=9) with the most severe form of disease (grade 4) as assessed by ultrasonography/angiography had increased TAT and d-dimer levels when compared with patients (n=27) having less severe disease (grades 1 through 3; Fig 2). The age- and sex-matched control subjects (n=20) had significantly lower TAT and d-dimer levels than the patients with mild disease (grades 1 through 3).
Discussion

In stable atherosclerosis of the large lower-limb arteries, we found a strong association between the functional severity of the disease and plasma fibrinogen. Fibrinogen has been established as an important independent cardiovascular risk factor for myocardial infarction, sudden coronary death, and stroke.19 Fibrinogen was the strongest independent predictor of death in stable claudicants who were prospectively followed up for 6 years.19 Elevated fibrinogen levels probably mediate the increased cardiovascular risk associated with smoking, the single most important risk factor for PAOD in about 90% of the patients.20,21 In the coronary arteries, the connection between fibrinogen and angiographically assessed atherosclerosis has not been uniformly observed in previous studies.22,23 The difference may be that the extent of atherosclerotic vessel wall damage is less prominent in CAD than in PAOD; ie, the potential thrombogenic surface is larger in PAOD. Also, angiographic analysis may be less useful in assessing the severity of atherosclerosis, since this procedure excludes functional effects on blood flow. ABI is considered the best clinical tool for assessing the severity of PAOD.24 And ABI predicts the local progression of the disease in follow-up studies.25,26 In our study, as also reported by others,27 ABI was associated not only with the local severity and extent of atherosclerosis but also with other cardiovascular manifestations, the main causes of excess mortality in PAOD.25,26 For the aforementioned reasons, we consider that this assessment of atherosclerosis provides a good reflection of both the extent of vascular damage in general and its local implications for blood flow.

The Clauss method may be disturbed by the presence of anticoagulant plasmin-generated fibrinogen degradation products, therefore yielding an underestimation of true fibrinogen levels. On the other hand, immunologic assays also include fibrinogen degradation products, leading to false high values.20 Because we detected an association between fibrinogen and D-dimer, we wanted to confirm the relation between functional fibrinogen and ABI, with fibrinogen assessed by an immunonephelometric technique (Table). We found an excellent correlation between fibrinogen values measured by these two techniques, and therefore, fibrinogen degradation products alone cannot account for the association with the severity of atherosclerosis. The observed relation between D-dimer and fibrinogen may depend on the stimulatory effect of plasmin-derived fragments of fibrinogen and fibrin on hepatic fibrinogen synthesis, which sustains increased fibrinogen levels.18,31 As to the elevated fibrinogen values, we deduced that overt infection was not the cause, since C-reactive protein levels were within normal limits in the majority of the patients, although they were elevated in 8. The present findings may represent the inflammatory process in the arterial wall.32

The increased levels of circulating split product of cross-linked D-dimer may originate from either the atherosclerotic surface or wall. Keen and Smith2 and Smith et al4 have reported that within the human atherosclerotic intima there is continuous formation of cross-linked fibrin and continuous fibrinolysis, which generates fragments with additional atherogenic properties. Furthermore, Bini et al5 have found fibrinogen/fibrin I and fibrin II surrounding vessel wall cells and macrophages in early lesions and fibrous plaques. Fibrin(ogen) degradation products were seen only in fibrous and advanced plaques together with fibrinogen/fibrin I and fibrin II. These pathological data fit well with our findings. In 60% of the patients with stable disease we also found signs of persistent thrombin formation, as reflected by the increased TAT and FpA levels in plasma. The generation of thrombin accords with enhanced platelet activation and abnormal vascular function, since increased excretion of thromboxane A2 and prostacyclin metabolites has been reported in severe atherosclerosis of lower-limb arteries.33 These signs of thrombin formation and enhanced platelet-vessel wall interaction support the concept that ongoing thrombogenesis is involved in the subclinical progression of atherosclerosis in large arteries. One possible mechanism for the locally enhanced fibrin deposition could be persistent procoagulant activity in the interface between flowing blood and the diseased vessel wall.34 In this setting, increased fibrinogen levels would promote increased fibrin turnover. Indeed, we found that TAT and D-dimer had a significant association (Table), and the patients having the most severe disease had the highest TAT and D-dimer values (Fig 2). Thrombin may also be constantly generated within the vessel wall and mediated by tissue factor, which is reported to be markedly expressed in atherosclerotic lesions and is likely to originate from inflammatory cells.35

In conclusion, the significant associations between the severity of atherosclerosis and circulating fibrinogen, as well as D-dimer, support previous pathological data that imply ongoing local degradation of cross-linked fibrin in the progression of atherosclerosis. The increased fibrinogen could enhance fibrin turnover in atherosclerotic vessels and vice versa; the high levels of plasmin-derived fibrin split products may increase synthesis of fibrinogen, a risk factor for acute thrombotic complications of atherosclerosis. Finally, the ongoing interaction between atherosclerotic vessel wall damage and activation of coagulation and fibrinolysis interferes with the applicability of the presented plasma markers during acute thrombosis.

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