Original Article

Fibrinogen Promotes Early Atherosclerotic Changes of the Carotid Artery in Young, Healthy Adults

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***Aim*: To determine whether high plasma levels or activities of different hemostatic proteins contribute to the development of early atherosclerotic vessel wall changes. Elevated levels of various hemostatic proteins and markers of inflammation have been linked to an increased risk of ischemic cardiovascular events; however, the mechanisms by which these molecules might contribute to this increased risk is not clear.**

***Methods*: The intima-media thickness of the common carotid arteries (CCA-IMT) of 125 healthy young volunteers without known cardiovascular risk factors was measured by high-resolution ultrasound. Plasma concentrations of fibrinogen, thrombomodulin, protein Z and CRP were quantified, and the plasma activities of protein C, plasminogen and factor Ⅷ were measured. Other established risk factors, such as body mass index (BMI) and plasma levels of cholesterol, triglycerides and homocysteine, were also determined. Furthermore, the carotid arteries were examined for the presence of plaques and stenoses.**

***Results*: Univariate analysis showed a significant negative correlation between CCA-IMT and HDL cholesterol, and positive correlations with age, BMI, LDL cholesterol, triglycerides, homocysteine, fibrinogen and thrombomodulin, but not with total cholesterol, lipoprotein(a) and hsCRP. CCA-IMT was also statistically independent of the activities of protein C, factor Ⅷ and plasminogen. Multivariate analysis revealed a significant correlation of CCA-IMT with age, BMI and fibrinogen.**

***Conclusion*: Our data suggest that fibrinogen levels correlate with early atherosclerotic changes of the carotid artery in a population with very low cardiovascular risk.**

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Key words; Hemostasis, Fibrinogen, Intima-media thickness, Atherosclerosis, Carotid artery, Ultrasound

Introduction

A number of proteins involved in hemostasis, inflammation, and endothelial damage are recognised as potential markers of elevated cardiovascular risk[1)]. The mechanisms underlying these associations may include the promotion of early and advanced atherosclerotic lesions as well as enhanced thrombus formation and plaque destabilisation[2-4)].

Common carotid artery intima-media thickness (CCA-IMT) is a widely accepted surrogate marker of early atherosclerosis, and increased CCA IMT has been linked to an elevated cardiovascular risk[5, 6)]; however, in the case of hemostatic proteins, different CCA-IMT studies have often yielded contradictory results. This might be because the activities, or plasma concentrations, of several potentially atherogenic hemostatic proteins can be influenced by other cardiovascular risk factors, such as obesity, smoking or arterial hypertension, which are all known, independent promoters of atherosclerosis. The presence of one, several, or all of these risk factors might produce variable

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biases in different studies of the relationship between hemostatic proteins and early atherosclerotic changes of the carotid arteries. A population of young and healthy volunteers without any known traditional cardiovascular risk factors, should therefore be studied to minimize the confounding effect of these non-hemostatic factors on CCA-IMT measurements.

To this end, we measured CCA-IMT in a group of 125 healthy young persons, who had been careful screened for the absence of cardiovascular risk factors, and correlated the results with measurements of several different hemostatic factors.

Materials and Methods

Study Population

The study was approved by the local ethics committee of our university and written informed consent was obtained from all study participants.

The study group consisted of 125 young and healthy volunteers. Persons <18 and >50 years of age, with a history of arterial hypertension, a resting blood pressure $>140/90$ mmHg, a history of hyperlipoproteinemia, LDL cholesterol >3.88 mmol/L or HDL cholesterol <1.16 mmol/L, fasting glucose >5.55 mmol/L or HbA1c $>6.0\%$, a history of diabetes mellitus, active smoking or any history of smoking, a family history of premature atherosclerotic disease or a body mass index >30 kg/m^2 were not eligible to participate in the study. A family history of premature atherosclerosis was defined as at least one first-degree relative with a history of myocardial infarction, stroke, coronary, cerebrovascular or peripheral artery disease before the age of 50 years. All persons entering the study were free of acute disease, especially inflammatory disease, and were not currently taking any medications.

Ultrasonography of Extracranial Vessels

High-resolution B-mode ultrasound, pulse-wave Doppler and colour-coded duplex ultrasound of both carotid arteries were performed using a iU22 ultrasound system (Philips Healthcare, NL) with a 9 MHz linear transducer. Patients were examined in the supine position. All ultrasound studies were performed by the same investigator, who was blinded to the laboratory values of the patients.

Carotid IMT was measured as described previously[7-9]. This method produces highly reproducible results (variation coefficient of 2.2% for intra-observer variability).

Briefly, CCA-IMT was measured from longitudinal images of 10 mm of the common carotid artery immediately proximal to the carotid bulb. Three angulations (anterior, lateral and postero-lateral) were obtained, and ten measurements of the IMT at the far wall of the artery were made from each angulation. All measurements were taken during the same time point in the cardiac cycle and at a plaque-free portion of the artery. Sixty measurements (30 from each side) were averaged to yield the mean IMT.

Furthermore, the presence of plaques (defined as localized thickening of >1.2 mm not involving the whole circumference of the artery) within the walls of both CCAs or stenoses of the extracranial vessels were noted.

Laboratory Tests

Serum and plasma samples were taken in the early morning after 8 hours of fasting, centrifuged and assayed immediately or frozen at -80℃.

Fibrinogen concentration was measured according to the modified Clauss method with a commercially available kit (Multifibren U; Siemens Healthcare, Bad Nauheim, Germany).

Tests to determine the concentrations of thrombomodulin (Asserchrom Thrombomodulin) and protein Z (Asserchrom Protein Z) were purchased from Roche Diagnostics (Mannheim, Germany); tests to determine the concentrations of hsCRP were purchased from Siemens Healthcare (Bad Nauheim, Germany).

Functional assays for protein C (Berichrom Protein C), plasminogen (Berichrom Plasminogen) and factor Ⅷ (Prothrombin SL) using factor Ⅷ-deficient plasma were performed on a BCS coagulation analyzer (all Siemens Healthcare, Bad Nauheim).

Lipoprotein(a) concentration was determined using a latex enhanced immunoturbidimetric assay (Scil Diagnostics, Martinsried, Germany). Total cholesterol, LDL- and HDL-cholesterol and triglycerides were measured with commercially available enzymatic-photometric assays, and homocysteine was measured with a competitive chemiluminescence immunoassay (all Siemens Healthcare, Bad Nauheim Germany).

Data Analysis

All descriptive data are presented as the mean ± standard deviation (S.D.). For all functional assays (protein C, factor Ⅷ, plasminogen), results are reported as a percentage of the median enzyme activity of a population of 500 healthy blood donors.

Linear regression analysis and Spearman's correlation analysis were performed to analyze the effects of biometric and biochemical data on CCA-IMT. For multivariate analysis, biometric and biochemical data

Table 1. Biometrical and biochemical data of the study population

female/male [n]	68/57
Age [years]	28.2 ± 6.0
BMI [kg/m^2]	23.1 ± 3.4
C-IMT [mm]	0,532 ± 0,064
Chol. [mmol/L]	4.55 ± 0.70
LDL [mmol/L]	2.47 ± 0.62
HDL [mmol/L]	1.61 ± 0.42
Trigl. [mmol/L]	0.95 ± 0.37
Hcy. [μmol/L]	10.5 ± 3.9
Fibrinogen [μmol/L]	7.83 ± 1.74
FVIIIc [%]	99.6 ± 19.5
Plasminogen [%]	103.6 ± 18.5
Thrombm. [ng/mL]	24.5 ± 8.3 (§9.9)
Protein C [%]	111.5 ± 18
Protein Z [ng/mL]	1,666 ± 594
Lp(a) [mg/L]	195 ± 191 (§99)
hsCRP [mg/L]	0.78 ± 0.76 (§0.16)

Abbreviations: BMI = body mass index, C-IMT = mean intima-media thickness of the common carotid artery, Chol = total cholesterol, LDL/HDL cholesterol = low/high density lipoprotein cholesterol, Trigl = triglycerids, Hcy = homocysteine, FVIIIc = factor VIII acticity, Thrombm. = Thrombomodulin, Lp(a) = Lipoprotein(a), hsCRP = high sensitive CRP, § = median.
Data are given as the means ± S.T.D

were entered into a logistic regression model. SPSS version 11.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis.

P values < 0.05 were regarded as significant.

Results

Biometric and biochemical data collected from the study population are presented in **Table 1**.

Univariate correlation analysis revealed a statistically significant positive correlation between IMT and age, body mass index (BMI), plasma concentrations of LDL cholesterol, triglycerides, homocysteine, fibrinogen and thrombomodulin, as well as a negative correlation with HDL levels, for the entire study population (**Table 2**). No significant correlation between CCA-IMT and gender, the plasma activity of plasminogen, factor VIII or protein C, or levels of protein Z, Lp(a) or hsCRP could be detected (**Table 2**).

When age, BMI, LDL-cholesterol, HDL-cholesterol, triglyceride, thrombomodulin, fibrinogen and homocysteine concentrations were analyzed with a multivariate logistic regression model, only age, BMI and fibrinogen levels were significantly correlated with CCA-IMT (**Table 3**).

Table 2. Univariate correlation coefficients of CCA-IMT and different biometrical and biochemical parameters of the study population ($n = 125$)

	Correlation coefficient	*p*
Age	0.471	< 0.001
BMI	0.385	< 0.001
Chol.	0.133	0.144
LDL	0.289	< 0.05
HDL	− 0.202	< 0.05
Trigl.	0.179	< 0.05
Homocystein	0.206	< 0.05
Fibrinogen	0.175	< 0.05
FVIIIc	− 0.068	0.447
Plasminogen	− 0.093	0.297
Thrombomodulin.	0.220	< 0.05
Protein C	0.133	0.136
Protein Z	− 0.082	0.361
Lp(a)	0.069	0.439
hsCRP	− 0.111	0.275

Abbreviations: BMI = body mass index, Chol = total cholesterol, LDL/HDL cholesterol = low/high density lipoprotein cholesterol, Trigl = triglycerids, FVIII = factor VIII acticity, Lp(a) = Lipoprotein(a), hsCRP = high sensitive CRP

No plaques or stenoses of the extracranial carotid vessels were detected in the study group.

Discussion

In this study we have demonstrated a positive association between plasma fibrinogen levels and CCA-IMT in a group of young healthy adults without relevant concomitant cardiovascular risk factors. By contrast, we could not show a significant association between CCA-IMT and soluble thrombomodulin, hsCRP, protein Z levels or protein C or factor VIII activities.

Previous studies of the relationship between the various hemostatic proteins measured, including fibrinogen, and early atherosclerotic changes of the extracranial carotid arteries have yielded inconsistent results, with some studies showing a significant correlation between fibrinogen and CCA-IMT [10-13] and some not [14-16]. Similarly conflicting results have been published for other hemostatic and inflammatory proteins measured in our study (protein C, factor VIII, thrombomodulin, hsCRP) [10, 11, 17-24].

There are, as highlighted in the "Introduction", important differences in the design of our study as compared to the studies mentioned above. Most other studies have investigated relatively inhomogeneous pa-

Table 3. Correlation of CCA-IMT and different biometrical and biochemical parameters of the study population ($n=125$), results of multivariate logistic regression analysis

	p
Age	<0.001
BMI	<0.05
Fibrinogen	<0.05
LDL	0.214
HDL	0.326
Trigl.	0.328
Thrombomodulin	0.385
Homocysteine	0.135

Abbreviations: BMI = body mass index, LDL/HDL = low/high density lipoprotein cholesterol, Trigl = triglycerids

tient cohorts, with a wide range of diseases and cardiovascular risk factors, while even those investigations targeting purportedly healthy volunteers have failed to exclude rigorously all individuals with hyperlipoproteinemia, arterial hypertension, a smoking history, or a family history of premature atherosclerosis. Similarly, subjects with inflammatory diseases, which can upregulate some hemostatic proteins, especially fibrinogen and factor Ⅷ, have also not been excluded in some of these studies. These problems are compounded by the fact that it nearly impossible to match subjects not only for the presence, but also for the duration and severity of each cardiovascular risk factor or inflammatory condition.

This lack of exclusion of subjects with pre-existing cardiovascular risk factors or inflammatory conditions is likely to account for at least some of the apparent contradictions between different studies. These factors can influence the concentrations and activities of the various hemostatic and inflammatory proteins, while also promoting early atherosclerosis independently, with resultant contributors to CCA-IMT. For example, fibrinogen levels as well as factor Ⅷ activities correlate positively with increasing age, waist to hip ratio and LDL-cholesterol levels. Also, smoking, metabolic syndrome and inflammation are associated with elevated plasma fibrinogen and factor Ⅷ concentrations[4, 13, 25, 26].

It has also been shown that a multivariate analytic approach is crucial in this setting, but was not performed in some previous studies[27].

The fact that we observed a correlation between plasma fibrinogen concentrations and CCA-IMT in our more carefully selected subjects therefore makes the strongest case yet for a causal link between fibrinogen and early atherosclerosis. This is further supported by fibrinogen, among all the tested hemostatic proteins, remaining as the only significant predictor of CCA-IMT in multivariate analysis.

Different mechanisms have been identified by which fibrinogen might promote atherosclerosis[2, 4]. Fibrinogen can bind to endothelial cell receptors (ICAM-1) and trigger the release of vasoactive mediators[28]. Fibrinogen and its degradation products modulate endothelial cell permeability, and promote endothelial cell migration[29, 30]. Fibrinogen has been shown to promote smooth muscle cell proliferation and induce monocyte chemotaxis[31, 32], and also may play a role in foam cell formation by the facilitation of cholesterol transfer from platelets to monocytes/macrophages[33, 34].

Our study does have certain limitations.

First, however carefully selected our study population was, the study sample size might be too small to detect small influences of hemostatic proteins and inflammation markers on CCA-IMT in a low risk population. However, even in our group of low-risk subjects, we were able to demonstrate a significant correlation between IMT and fibrinogen and other established cardiovascular risk factors, such as age and BMI. Also, significant differences in IMT between individuals with cardiovascular risk factors, such as diabetes mellitus, arterial hypertension and hypercholesterolemia (as single cardiovascular risk factors), and healthy controls have been demonstrated in other studies with a similar or even smaller number of participants[35, 36].

A second limitation is that we did not include a glucose challenge test or repeated blood pressure monitoring to exclude diabetes mellitus and arterial hypertension, respectively; however, we strictly excluded persons with any history of medical abnormalities and therefore regard the potential subsequent bias as minimal.

Conclusion

In summary, we found a significant correlation between fibrinogen levels and early atherosclerotic changes of the common carotid artery in a group of young, healthy persons without concomitant cardiovascular risk factors. Our data suggest that in these subjects with very low cardiovascular risk, fibrinogen levels may serve as early and highly valid predictors for the development of atherosclerotic disease and that fibrinogen may contribute to early atherosclerotic changes of the carotid arteries.

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