

ORIGINAL RESEARCH

Factor VII Activating Protease Expression in Human Platelets and Accumulation in Symptomatic Carotid Plaque

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BACKGROUND: Factor VII activating protease (FSAP) is of interest as a marker for vascular inflammation and plaque destabilization. The aim of this study was to analyze the expression profile of FSAP in endarterectomy specimens that were taken from patients with asymptomatic and symptomatic carotid atherosclerotic plaques and to compare them with circulating FSAP levels.

METHODS AND RESULTS: Plasma FSAP concentration, activity, and mRNA expression were measured in endarterectomy specimens and in monocytes and platelets. Plaque and plasma FSAP levels were higher in symptomatic patients (n=10) than in asymptomatic patients (n=14). Stronger FSAP immunostaining was observed in advanced symptomatic lesions, in intraplaque hemorrhage-related structures, and in lipid-rich areas within the necrotic core. FSAP was also colocalized with monocytes and macrophages (CD11b/CD68-positive cells) and platelets (CD41-positive cells) of the plaques. Moreover, human platelets expressed FSAP in vitro, at both the mRNA and protein levels. Expression is stimulated by thrombin receptor-activating peptide and ADP and reduced by acetylsalicylic acid.

CONCLUSIONS: Plasma FSAP levels were significantly increased in patients with symptomatic carotid stenosis and thus may be involved in plaque development. This plaque-associated FSAP may be produced by platelets or macrophages or may be taken up from the circulation. To establish FSAP's utility as a circulating or plaque biomarker in patients with symptomatic carotid atherosclerotic plaques, further studies are needed.

Key Words: asymptomatic carotid stenosis ■ atherosclerosis ■ FSAP ■ inflammation ■ platelet ■ symptomatic carotid stenosis

Atherosclerosis is an inflammatory disorder that can develop into an acute cardiovascular event through plaque vulnerability, rupture, and thrombosis.¹ Factors that can predict evolution of plaque vulnerability can improve risk prediction. Increasing evidence supports a role of factor VII activating protease (FSAP) in regulating a variety of processes related to plaque vulnerability and rupture.^{2–6} Furthermore, recent data indicate its involvement in several other pathways related to endothelial permeability, vascular remodeling, and inflammation.^{7–11} With respect to

coagulation and fibrinolysis, a number of substrates for FSAP have been identified such as factor VII, fibrinogen, single-chain plasminogen activators (pro-urokinase) and tissue factor pathway inhibitor.^{12–16} Our earlier investigations indicated that FSAP is present in human coronary atherosclerotic plaques from patients with stable or unstable angina and/or myocardial infarction but not in normal vessels.¹⁷ The staining of FSAP in unstable coronary plaques was associated with cells positive for the macrophage-specific marker CD68.¹⁷ In addition, a variant in the FSAP gene, Marburg I, is

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CLINICAL PERSPECTIVE

What Is New?

- Factor VII activating protease (FSAP), a marker of inflammation and plaque destabilization, is involved in the development of symptomatic carotid plaque stenosis.
- According to our study data, we could show that monocytes, macrophages, and platelets are the main source of FSAP, as it is colocalized in CD41/CD68-rich plaque regions with increasing levels of CD41 in vulnerable plaques.
- Our statement can be further validated by the fact that FSAP protein level increases during plaque development.

What Are the Clinical Implications?

- FSAP might be seen as a new actor in the evolution of carotid atherosclerotic disease, representing a new target of therapeutic approaches in vascular proliferative diseases.

Nonstandard Abbreviations and Acronyms

aCAS	asymptomatic carotid artery stenosis
ASA	acetylsalicylic acid
FSAP	factor VII activating protease
sCAS	symptomatic carotid artery stenosis
TRAP	Thrombin receptor activating peptide

associated with advanced carotid stenosis, cardiovascular risk, and stroke.^{18–20} Although we could show that the Marburg I and II FSAP gene variants are not predictive or prognostic biomarkers for coronary artery disease, the presence of the Marburg II variant was associated with a reduced risk of developing coronary artery disease.²¹

Taken together, these data support the hypothesis that FSAP could have a regulatory role during human atherosclerotic plaque progression and vulnerability. In this study, we evaluated FSAP levels in plasma and FSAP expression patterns in human asymptomatic and symptomatic carotid plaques. Nonatherosclerotic specimens were obtained from internal mammary arteries and served as controls. Freshly isolated platelets and monocytes were analyzed for FSAP mRNA and protein. The role of these blood cells as sources of FSAP was further characterized *in vitro*. Finally, this study was designed to investigate whether plaque FSAP levels correlate with clinically relevant stages of human atherosclerotic disease. This may help to develop a biomarker for plaque progression and vulnerability.

METHODS

Data, Materials, and Code Disclosure Statement

The data that support the findings of this study are available from the corresponding author on request.

Study Population

During a period of 14 months, 50 patients with high-grade carotid stenosis admitted to the Department of Vascular Surgery, University Hospital of Giessen and Marburg, were enrolled in the single-center study, whereas only 39 patients fully satisfied the enrollment criteria. On the basis of their clinical characteristics, patients were classified into 2 diagnostic groups—patients with asymptomatic carotid artery stenosis (aCAS, n=14) and patients with recently high-grade symptomatic CAS (sCAS; 60%–90%, n=10)—and nonatherosclerotic controls (ie, patients without CAS or coronary artery disease, n=10; age- and sex-matched) for inclusion in this study. Age and sex, cardiovascular risk factors, including systemic arterial hypertension, diabetes mellitus, smoking, serum characteristics (eg, dyslipidemia), family history of coronary artery disease, and medication were assessed and recorded at study entry (Table 1). This study was approved by the ethics committee of the University of Giessen, and written informed consent was obtained from all participants.

Presence of sCAS was defined as being referred to the stroke unit for symptoms of transient ischemic attack or stroke with high-grade CAS (60%–90%). aCAS is defined as a $\geq 70\%$ narrowing of the carotid artery in the

Table 1. Patient Characteristics

Characteristic	Controls	Asymptomatic	Symptomatic
	(n=15)	(n=14)	(n=10)
Age, y (arithmetic average)	63	67	69
Female sex, n (%)	4 (27)	4 (28)	3 (30)
Cardiovascular risk factors, n (%)			
Smoking	4 (27)	4 (28)	4 (40)
Arterial hypertension	1 (7)	11 (78)	10 (100)
Dyslipidemia	1 (7)	9 (64)	6 (60)
Diabetes mellitus	0 (0)	3 (21)	2 (20)
Positive FH	0 (0)	4 (28)	1 (10)
Medical treatment, n (%)			
Aspirin/clopidogrel	0 (0)	14 (100)	10 (100)
Statin	0 (0)	14 (100)	6 (60)
ACEI/ARB	1 (7)	11 (78)	8 (80)
β -Blocker	0 (0)	9 (64)	3 (30)

ACEI indicates angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; and FH, family history.

absence of retinal or cerebral ischemia in the preceding 6 months. Treatment of the patients was according to the hospital's standard routine following the guidelines on the diagnosis and treatment of peripheral arterial diseases.²² At admission, ultrasound examinations to determine the degree of CAS and following a cerebral computed tomography scan were performed to verify the location and extent of a carotid stenosis or occlusion and to exclude intracerebral hemorrhage. Chronic small vessel disease (cerebral microangiopathy) was defined with occurrence of lacunar lesions in the subcortical layer and according to the patient's clinical picture at admission. Diffusion-weighted sequences on magnetic resonance imaging were performed to reliably detect or exclude acute lacunar lesions. Furthermore, patients were asked for cardiac arrhythmia events during past 6 months and underwent ECG diagnostics and echocardiography for the screening of atrial fibrillation and to exclude cardiac thromboembolism.

All patients with sCAS underwent carotid endarterectomy as soon as they were considered stable by the stroke unit. All tissue samples were immediately snap frozen in liquid nitrogen and stored at -80°C until use. Furthermore, 15 unaffected internal mammary arteries were obtained during coronary bypass surgery and were used as control for the immunohistochemical tests.

Entry criteria were as follows: (1) a transient ischemic attack or stroke ≤ 6 weeks before carotid endarterectomy, (2) no previous neurologic symptoms before carotid endarterectomy, and (3) internal or common carotid artery stenosis $>70\%$ defined by velocity criteria on duplex scanning. Exclusion criteria for patients were (1) amaurosis fugax alone or nonfocal, atypical, or distant neurologic symptoms; (2) recent (<6 months) carotid endarterectomy; (3) recent (<6 months) symptoms of transient ischemic attack and stroke; (4) pregnancy or breastfeeding; (5) inability to provide written informed consent or rejection to participate; (6) contraindications for carotid endarterectomy; (7) body temperature $>38.0^{\circ}\text{C}$ with inflammatory disease (eg, infections, autoimmune diseases); (8) malignancies; (9) atrial fibrillation with a reason for cardioembolism; (10) small vessel disease (lacunes); and (11) intracerebral hemorrhage.

FSAP Concentration and Activity

Immunocapture activity test using a monoclonal antibody against FSAP was used to measure the total FSAP activity.^{5,23} The total FSAP concentration was detected by FSAP-specific ELISA. For FSAP activity assays, microtiter plates were coated with anti-FSAP mouse monoclonal antibody 677 (American Diagnostica) and coating buffer. Diluted plasma probes were applied to the plate, which was incubated for 1 hour at room temperature and then

washed 3 times. Recombinant scuPa (Saruplase) and chromogenic substrate S-2444 (Haemochrome) were added and incubated (37°C), and the absorption at 405 nm was recorded with a microplate reader EL 808 (Biotek Instruments). Calibration curves were established through dilution series of standard human plasma (Siemens Medical Solutions and Diagnostics) and served as the reference for measured FSAP activity. The plasma activity in 1 mL standard human plasma was defined as 1 plasma-equivalent U/mL. To detect the total plasma FSAP concentration, microtiter plates were coated with a rabbit polyclonal anti-FSAP antibody (American Diagnostica), and diluted patient plasma was then incubated for 1 hour at room temperature. After extensive washing, anti-FSAP monoclonal antibody 677 was added and incubated (60 minutes at room temperature). Detection was performed with a peroxidase-coupled mouse antibody and 3,3',5,5'-tetramethylbenzidine (TMB-Substrate-Kit; Pierce). Optical density was measured at 405 nm, and the FSAP concentration was quoted in micrograms per milliliter using standard human plasma.

Tissue Preparations and Histologic and Immunohistochemical Analyses

Carotid specimens and internal mammary artery specimens were prepared as described previously.¹⁷ Briefly, all tissue samples were embedded in Tissue Tek mounting medium (Sakura Finetek) and shock-frozen in liquid nitrogen before they were cut and prepared for further analysis. Afterward, they were serially sectioned in 6- μm -thick slices using the CM 1900 cryostat (Leica). Tissue sections were collected on glass slides, air-dried, and fixed in cold acetone at 4°C for 10 minutes. The LSAB staining procedure was performed using HRP-AEC Cell and Tissue Staining Kits (R&D Systems), and all steps were carried out according to the manufacturer's instructions. Before staining, slides were fixed in ice-cold acetone for 20 minutes and then air-dried for better adherence of the slices. To avoid any unwanted nonspecific binding, sections were incubated with diluted mouse serum (1:3000, 20 minutes) followed by peroxidase-, serum-, avidin-, and biotin-blocking reagents (ready-to-use solutions, 15 minutes each). Between the blocking steps, sections were carefully rinsed with PBS for 15 minutes. Subsequently, diluted monoclonal primary antibodies were used to incubate all samples overnight in a humidified chamber at 4°C . The primary antibodies used in this study were mouse monoclonal antibodies against FSAP light chain (Behring), CD68 (EBM11)/CD11b (2LMP19) for identification of monocytes/macrophages, CD41 (platelet glycoprotein IIb) for identification of platelets (all from Dako). After further washing steps with PBS (3×15 minutes),

all slices underwent the next incubation step (60 minutes) with a secondary biotinylated antibody directed against the Fc-part of the primary antibody. The sections were incubated another 30 minutes at room temperature with high-sensitivity-streptavidin molecules linked to a horseradish peroxidase enzyme. The activity of the horseradish peroxidase enzyme was visualized with AEC (3'-amino-9'-ethylcarbazol), which, after an incubation step of 20 minutes, yielded red staining results among positive areas in the carotid atherosclerotic plaques. The chromogen was first diluted in a specific chromogen buffer from the staining kit and then trickled on the slides at an approximate volume of 50 μ L per slice. Finally, all slides were gently rinsed with distilled water and then counterstained with hematoxylin, which contrasted positive red staining results very well. Hematoxylin–eosin stained sections from each tissue block were examined to establish the morphologic characteristics of the plaques, in accordance with the classification of Stary.²⁴ Negative control sections were prepared from the same tissue block, but primary antibodies were substituted by normal control ones without any immunogenicity (data not shown). The scoring of positive FSAP staining was based on positive areas per plaque and on the density of a positive staining per area, as described.¹⁷ A scoring degree was made using a scale of 0 (negative), 1 (weak positive staining), and 2 (strong positive staining) (Table 2). The microscopic evaluation and quantitative analysis of the sections was done by at least 2 investigators who were blinded to the patients' characteristics and histologic classifications.

Isolation of Human Monocytes

Monocytes from all patients and nonatherosclerotic participants were isolated with the RosetteSep Kit, according to the manufacturer's instructions and as described previously.⁵

Preparation of Human Platelet Isolation

Human platelets were taken from healthy young male donors fasting at least 6 hours before the intervention. The blood withdrawal from the cubital vein was performed using specific tubes at room temperature, containing a mixture of the 4 anticoagulant substances (citrate, theophylline, adenosine, and dipyridamole; Vacutainer CTAD [BD]) to prevent immediate clotting and coagulation of the blood samples. To obtain sufficient amounts of isolated platelets, 50 mL of blood was drawn from every donor and centrifuged at 72g for 20 minutes. The platelet-rich supernatant was placed into new 15-mL tubes (Falcon). Platelets underwent a washing step by adding the Tyrode's buffer pH 6.5 at a 1:1 ratio combined with a second centrifugation

Table 2. Carotid Endarterectomy Samples: Characteristics of Lesion

No.	Location of Lesion	Diagnosis	FSAP Antigen
1	Right	Asymptomatic	1
2	Right	Asymptomatic	1
3	Left	Asymptomatic	0
4	Right	Symptomatic	2
5	Left	Symptomatic	2
6	Left	Asymptomatic	0
7	Right	Symptomatic	2
8	Left	Symptomatic	2
9	Left	Asymptomatic	0
10	Right	Asymptomatic	1
11	Right	Asymptomatic	1
12	Left	Asymptomatic	0
13	Left	Asymptomatic	1
14	Left	Symptomatic	1
15	right	Symptomatic	2
16	Left	Symptomatic	2
17	Right	Asymptomatic	1
18	Right	Asymptomatic	1
19	Left	Asymptomatic	0
20	Left	Symptomatic	2
21	Right	Asymptomatic	0
22	Right	Symptomatic	1
23	Left	Symptomatic	2
24	Left	Asymptomatic	1

Scale: 0, no detectable staining; 1, weak positive staining; 2, strong positive staining. FSAP indicates factor VII activating protease.

procedure at 392g for 15 minutes. Washed platelets were isolated from platelet-rich plasma, which was obtained by centrifugation of whole blood at 120g for 15 minutes at 20°C. Platelet-rich plasma was decanted and acidified to pH 6.5. Apyrase (25 μ g/mL) and prostaglandin E1 (100 nmol/L) were added. The platelets were resuspended in washing buffer. To exclude any contamination with white blood cells (eg, monocytes) and to determine the approximate amount of platelets ($\approx 1800 \times 10^9$ /L), we finally analyzed 100 μ L of each solution before the stimulation. The platelets did not exhibit any monocyte contamination, as indicated by flow cytometry using anti-CD14 antibodies. To avoid any unwanted cell activation or thermic stress, all reagents were preheated, and tubes were strictly kept in a ThermoCycler at 37°C during incubation times. A suspension (in Tyrode's buffer) of 70×10^6 freshly isolated platelets was placed in centrifugation tubes. To obtain activated platelets, we used ADP (10 μ mol/L per mL) and thrombin receptor-activating peptide (TRAP; 5 μ mol/L per mL). In addition, some specimens were preincubated with aspirin or acetylsalicylic acid (ASA; 100 μ mol/L per mL) for 15 minutes to show putative

reduced activation of the platelets. Cells incubated in medium (Tyrode's buffer) alone served as a control group. Shortly after the incubation, all tubes were gently centrifuged at 1008g for 5 minutes. Thereafter, the supernatant was removed, and the pellet was prepared for FSAP expression analysis using real-time reverse transcription polymerase chain reaction (RT-PCR), Western blot, and immunocytochemical techniques.

RNA Isolation and Real-Time RT-PCR

Total RNA was isolated using the RNeasy Kit (Qiagen), according to the manufacturer's instructions, including DNase treatment. cDNA synthesis was performed using a kit from Perkin Elmer, and the cDNA was stored at -20°C , as described previously.^{5,10,17} RNA concentrations were measured using an Eppendorf BioPhotometer. Relative mRNA quantification was performed by real-time RT-PCR using a CFX 96 real-time system (Bio-Rad). For FSAP, the following primer sequences were used: sense, CCCACTGAGCCATCAACCAA; antisense, TGGGGCATGGAGATGGTC with a product size of 169 bp. The expression was normalized to the internal control (GAPDH; sense, CCT CAA GAT CAT CAG CAA TGC CTC CT; antisense, GGT CAT GAG TCC TTC CAC GAT ACC AA) and, relative to the expression in control group, was calculated. Relative mRNA quantification was performed by real-time PCR using the Sequence Detection System 7700 (PE Applied Biosystem). The expression of FSAP mRNA was normalized to GAPDH mRNA using the following equation: $T_0/R_0=2^{(CT,R-CT,T)}$, where T_0 is the initial number of target gene copies; R_0 , the initial number of standard gene copies; CT,T , the threshold cycle of the target gene; CT,R , are the threshold cycle of the standard gene, as described previously.^{6,22}

Western Blot

Western blot analysis of cell lysates was performed as previously described.¹⁰ For protein detection, specific antibodies against FSAP (Behring) and GAPDH (Calbiochem) were used.

Biochemical Evaluation of Platelet-Activation Markers

Levels of soluble CD62P and CD40L were analyzed in duplicate using commercial enzyme-linked immunosorbent assay kits and reagents (R&D Systems), according to the manufacturer's recommendations.

Immunofluorescence

Immunofluorescence was performed to evaluate intracellular FSAP antigen in stimulated platelets from healthy donors. Cytopins were prepared with

isolated platelets (≈ 1 million cells per slide). Cells were fixed with 4°C acetone and air-dried for 5 minutes, followed by a blocking step with goat serum (dilution range 1:1000). All slides were gently rinsed in PBS for a further 5 minutes before the cells were incubated with the first antibodies. To minimize un-specific background staining, primary antibodies were diluted in PBS supplemented with 1% BSA, and slides were then incubated for 2.5 hours in a humidified chamber. After further washing steps with PBS, second antibodies that were diluted in antibody diluents were added. Thereafter, slides were again put in humidified and dark chambers for 1 hour at room temperature. Double staining was performed to identify intracellular FSAP antigen and platelets (CD41). Negative controls were conducted by substituting the primary antibody through an isotype control one. The sections were washed and mounted in water-soluble Mowiol. Slides were covered with cover glasses and prepared for analysis using fluorescent light microscopy.

Determination of Marburg I Single-Nucleotide Variant

Genomic DNA was prepared from frozen whole blood with the GenomicPrep blood DNA isolation kit (Amersham Pharmacia Biotech), and PCR was performed, as described previously.^{5,21,25}

Statistical Analysis

All results for continuous variables are expressed as mean \pm SD or median (interquartile range), and categorical variables are shown as numbers and percentages. Data are presented in box plots with median (25th–75th percentiles) and whiskers (10th–90th percentiles). Data were found to be not normally distributed according to D'Agostino and Pearson omnibus normality test and were compared using the Kruskal–Wallis test followed by Dunn corrections for multiple comparisons. Differences between asymptomatic and symptomatic patient groups were compared with an unpaired Student *t* test or Mann–Whitney *U* test when the distribution was not normal. All statistical calculations were performed using the statistical package GraphPad Prism v6.05 (GraphPad Software). All hypotheses were 2-tailed, and $P\leq 0.05$ was considered statistically significant.

RESULTS

Plasma FSAP Level and Activity in Patients With aCAS and sCAS

There were no significant differences in the baseline clinical characteristics of the patients with aCAS

and sCAS (Table 1). However, 4 patients in the sCAS did not take statins, because of side effects and intolerance. Both FSAP antigen levels and activity in plasma increased significantly in patients with aCAS and reached maximum levels in patients with sCAS (Figure 1A and 1B). Furthermore, circulating monocytes of patients with aCAS and sCAS showed increased FSAP mRNA expression compared with healthy control participants (Figure 1C). We also evaluated the association of FSAP gene variants (Marburg I or II) with sCAS in the cluster of 24 patients. Only 1 patient was heterozygous for the FSAP Marburg I variant, corresponding to a carriage rate of 4.2%.

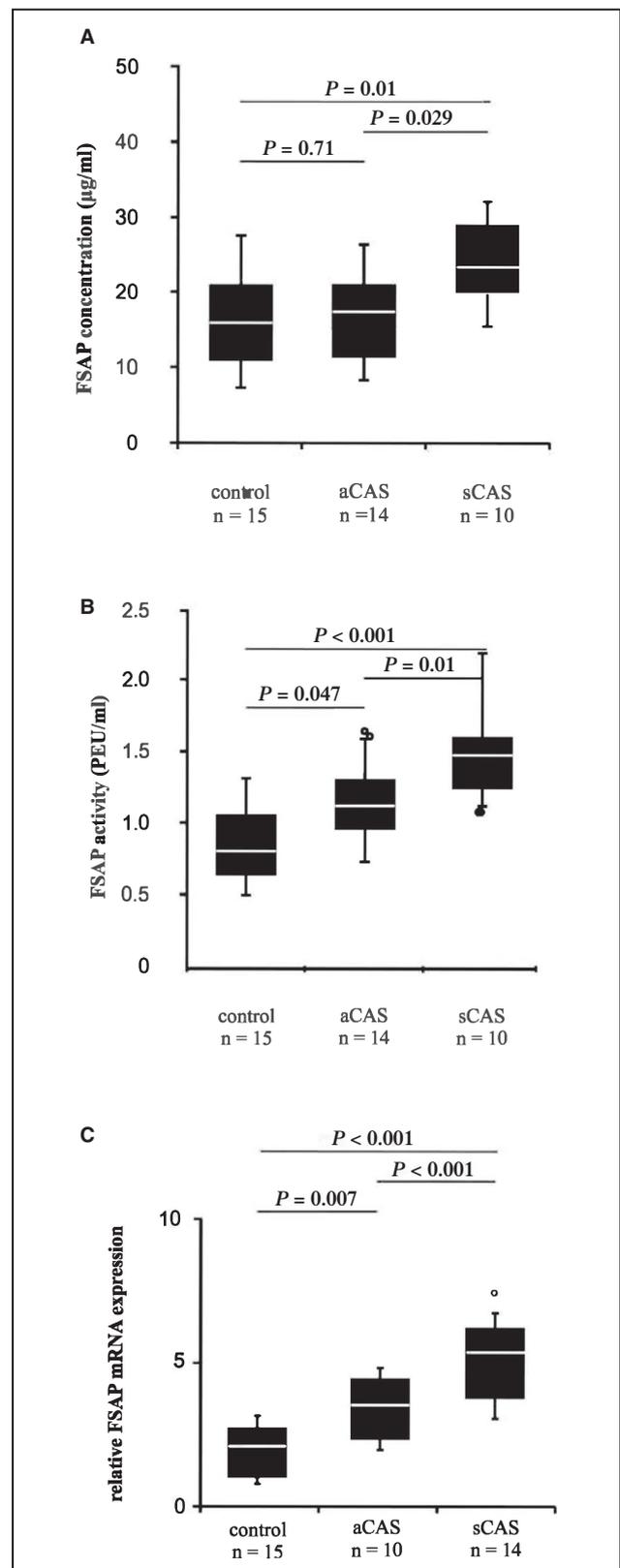
FSAP Expression in Asymptomatic and Symptomatic Human Carotid Atherosclerotic Plaques

Quantitative analysis of FSAP immunostaining in all human carotid atherosclerotic plaques, classified by histologic examination after staining with hematoxylin–eosin, showed that positive staining for FSAP was observed in both asymptomatic and symptomatic carotid plaques (Table 2). However, stronger expression and higher positive FSAP immunostaining (10/10 cases) was detected in symptomatic carotid plaques compared with asymptomatic carotid plaques (6/14 cases; Table 2). Results could be confirmed by real-time RT-PCR, which demonstrated the presence of higher levels of FSAP mRNA in symptomatic versus asymptomatic carotid plaques ($P < 0.01$; Figure 2C).

Serial immunologic analysis of the carotid lesions showed that the majority of FSAP-positive cells were also positive for the macrophage marker CD68 and the platelet marker CD41 (Figure 2A and 2B). Only a few macrophages infiltrated the vessel wall in early plaques; in advanced lesions, FSAP antigen was localized

Figure 1. Changes in the plasma concentration and activity level of factor VII activating protease (FSAP) during different stages of atherosclerosis development.

Box/whisker plots of FSAP concentration (A) and activity level (B) in patients with different stages of atherosclerosis development: control population, patients with asymptomatic carotid artery stenosis (aCAS), and patients with symptomatic carotid artery stenosis (sCAS). Time course of increased FSAP gene expression in monocytes from patients with aCAS and sCAS (C). FSAP mRNA levels were determined by real-time reverse transcription polymerase chain reaction. Relative mRNA quantification was performed and the fold change in the target gene (FSAP), normalized to the internal control (GAPDH) and relative to the expression in the control group, was calculated and presented as a box/whisker plot. Boxes mark the 25th and 75th percentile values, the line inside each box is the median, and whiskers indicate the spread of the 5th and 95th percentiles. Significance was assumed at $P \leq 0.05$, compared with the control group.



mainly in inflammatory regions within the shoulders of the plaque, the lipid core, and the fibrotic cap due to infiltration by macrophages (Figure 2B). No staining was found in normal nonatherosclerotic arteries (data not

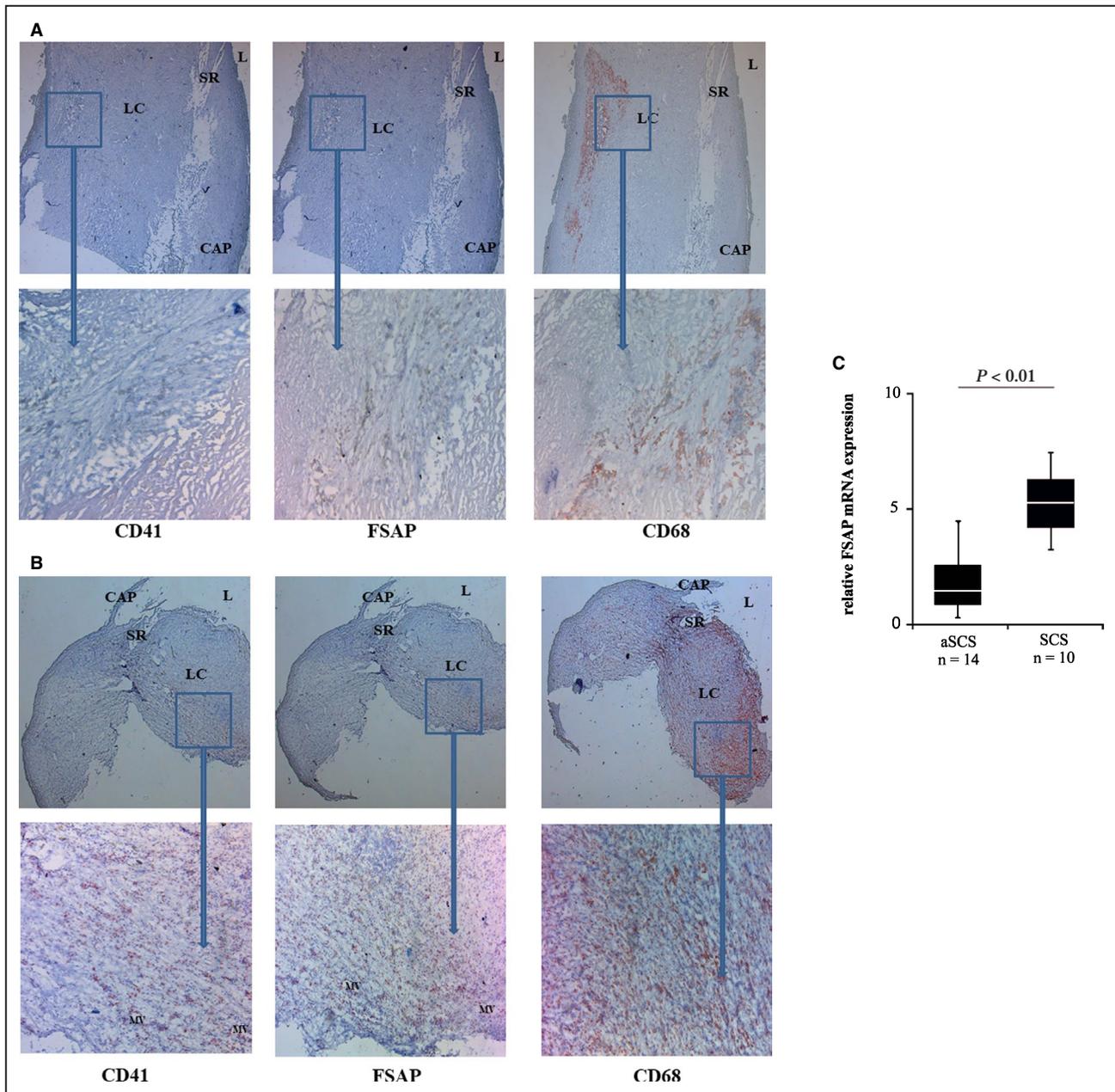


Figure 2. Comparative immunohistochemical localization of factor VII activating protease (FSAP) in human carotid atherosclerotic plaques.

Representative consecutive frozen sections from early asymptomatic (**A**) and advanced symptomatic (**B**) carotid plaques were stained immunohistochemically with antibodies against FSAP, CD68, and CD41. Positive staining for FSAP was more frequent in symptomatic carotid plaques and particularly colocalized with macrophages and platelets antigen expression in the shoulder region of the plaque. FSAP antigen was detected in the extracellular lipid-rich areas within the lipid core of the lesions. In addition, platelets were localized around microvessels at the border of the atheroma and in the lipid core of an atherosclerotic plaque. In both (**A** and **B**), the upper panel is $\times 100$; high-magnification ($\times 200$) views of the lesion area specified with rectangular boxes in the upper panel are shown in the lower panel. Fibrous cap (CAP), lumen (L), lipid core (LC), shoulder region (SR), and microvessels (MV) are indicated. **C**, FSAP mRNA expression in carotid atherosclerotic specimens. Boxplot analysis of normalized FSAP mRNA in asymptomatic and symptomatic atherectomy samples. Relative FSAP mRNA expression was significantly enhanced in plaques from patients with sCAS compared with those from patients with aCAS. Values are expressed as the ratio of the copy number of the gene of interest (FSAP) to the copy number of housekeeping gene (GAPDH). The fold change was calculated for each group. Data represent mean \pm SD, and significance was assumed at $P \leq 0.05$.

shown). In addition, immunohistochemical analysis for FSAP and the platelet-specific marker CD41 showed that early asymptomatic lesions were negative for

CD41 (Figure 2A), whereas some areas within symptomatic plaques were clearly positive for CD41, indicating the infiltration of platelets (Figure 2B). Tissues

showed no staining with the respective control IgG (data not shown).

FSAP Is Expressed in Isolated Platelets

The results reported suggested that platelets could represent a source of FSAP in carotid atherosclerotic lesions. Because platelets are anuclear cells that inherit their mRNA from megakaryocyte precursors and maintain it unchanged during their life span, gene expression profiling at the time of a transient ischemic attack or stroke provides information concerning the platelet gene expression preceding the acute carotid artery event.²⁶ We thus investigated FSAP expression in highly purified platelets isolated from human blood.

The presence of FSAP transcripts in platelet mRNA was observed by real-time RT-PCR and by Western blot and immunofluorescence (Figure 3A through 3C). The absence of contaminating monocytes was indicated by the fact that CD14 was undetectable in isolated platelets by flow cytometry and real-time RT-PCR (data not shown). Because FSAP was highly expressed in platelets of carotid atherosclerotic lesions, we tested whether mediators of activated platelets might up-regulate FSAP expression. Interestingly, stimulation of platelets with ADP and TRAP resulted in a 4.0-fold and 2.5-fold increase, respectively, of FSAP mRNA expression (Figure 3A through 3C), indicating that FSAP expression is induced during platelets activation. There were no differences in values of studied platelet-related

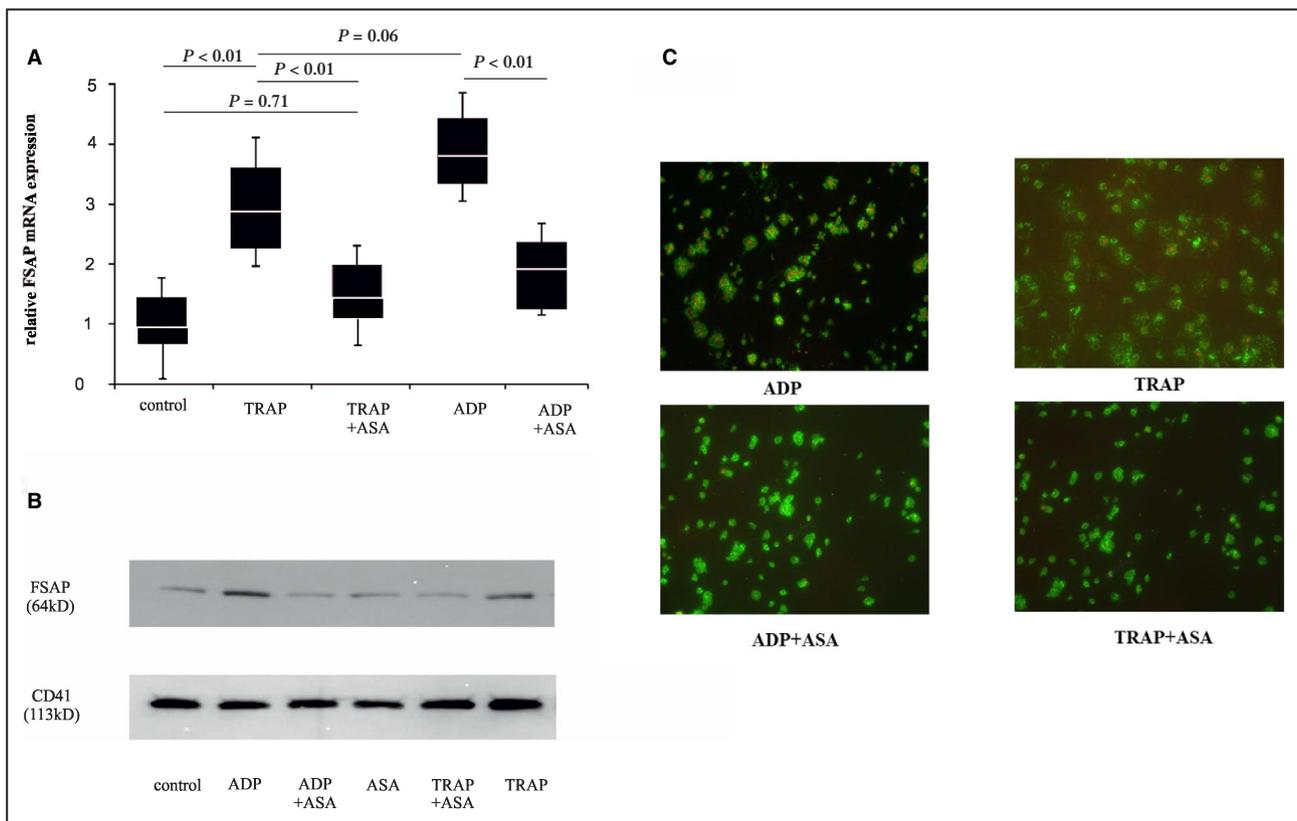


Figure 3. Factor VII activating protease (FSAP) expression in human platelets.

(A) ADP and thrombin receptor-activating peptide (TRAP) induce FSAP gene upregulation in freshly isolated human platelets. Platelets cultured in vitro were incubated with medium alone (control), 10 $\mu\text{mol/L}$ per mL ADP or 5 $\mu\text{mol/L}$ per mL TRAP for 15 minutes. In addition, some specimens were preincubated with aspirin or acetylsalicylic acid (ASA; 100 $\mu\text{mol/L}$ per mL) for 15 minutes to show putative reduced activation of the platelets. Relative mRNA quantification was performed, and the fold change in the target gene (FSAP), normalized to the internal control (GAPDH) and relative to the expression in the control group, was calculated and presented as a box/whisker plot. Boxes mark the 25th and 75th percentile values, the line inside each box is the median, and whiskers indicate the spread of the 5th and 95th percentiles. Significance was assumed at $P \leq 0.05$, compared with the control group. (B) Representative gels of FSAP protein. Platelets were treated with ADP or TRAP in the presence and absence of ASA. Cytoplasmic protein extracts were prepared and immunoblotting was performed for FSAP (upper panel), whereas CD41 was used as a loading control (bottom panel). Each panel is representative of at least 5 independent experiments performed in separate cell isolates. Significance was assumed at $P \leq 0.05$, compared with the control group. (C) Effect of ASA on visualization of FSAP in ADP- and TRAP-activated platelets in the absence (upper panel) and presence (bottom panel) of ASA. Activated platelets (CD62P, green and FSAP, red; merge upper panel). Same microphotographs of platelets with CD62P and FSAP in the presence of ASA (bottom panel) were visualized by immunofluorescence. The decrease of fluorescence density of FSAP shows the influence of the ASA on the FSAP expression. Similar findings were obtained from the samples prepared from the other 3 donors. Bar=10 μm .

inflammatory biomarkers (CD62P and CD40L surface expression) in both ADP platelets and those activated with TRAP (data not shown). ASA as a standard primary and secondary medication in patients with clinical manifestations of atherosclerotic disease such as stroke, and myocardial infarction was associated with significantly reduced FSAP expression in ADP- and TRAP-induced activated platelets (Figure 3A through 3C).²⁷

DISCUSSION

The contribution of FSAP to atherosclerosis and atherothrombosis has been largely studied but remains ambiguous. This study shows that FSAP expression in carotid plaques, circulating monocytes, platelets, and plasma is associated with the appearance of atherothrombosis under vulnerable inflammatory conditions. This strengthens the hypothesis that FSAP is a marker of sCAS. With regard to this aspect, the activation of circulating monocytes during vascular inflammatory processes, associated with an increased FSAP expression and activation, may contribute to the pathogenesis of transient ischemic attack or stroke as clinical manifestations of atherosclerotic disease, plaque rupture, and finally plaque thrombosis. Of mention in this context are factors like polyphosphates, polyamines, and nucleic acids, which activate FSAP and are produced by the inflammatory vasculature and systemic inflammatory state in patients with cardiovascular diseases.²⁸ However, we found that a higher level of FSAP activity was not accompanied by higher plasma FSAP concentration in patients with aCAS.

The most significant observation of this study was to show that monocytes and macrophages as well as platelets are the main sources of FSAP in human symptomatic carotid plaques, as indicated by the colocalization of FSAP and CD68/CD41-positive cells, respectively. The relationship between enhanced plaque expression of FSAP and increased plasma FSAP levels in clinically relevant CAS was further supported by our analyses showing increased FSAP concentration and activity in these patients as an important feature during the development of this unstable disease. Furthermore, immunohistochemical studies also indicate that the presence of FSAP protein within lesions globally increases with plaque development—from stable asymptomatic carotid plaques to advanced symptomatic carotid plaques. These data are in line with the results of our previous studies of coronary atherosclerotic plaques, showing FSAP present in inflammatory cells, influencing their activation due to increased expression levels, which were further induced by proinflammatory mediators.¹⁷

Thus, FSAP contributes to the atherosclerotic inflammatory process through different actions and has gained interest as a possible marker for human vulnerable plaques.¹⁷

Both plaque and plasma levels of FSAP were increased in patients with sCAS, whereas no significant associations were found in aCAS. This finding could correspond to a release into the plaque–media–adventitia rather than to the lumen–circulation in human atherosclerosis, resulting in increased FSAP levels in the plaque, which is not reflected in plasma. Nevertheless, in the case of a fibrous cap rupture, blood penetrates the tissue and plasma levels of FSAP increase, according to our results.

This study is the first to show that plaque FSAP is also colocalized with areas positive for the platelet antigen CD41 in a vulnerable phenotype of symptomatic carotid plaque. However, early asymptomatic carotid lesions were negative for CD41. Moreover, FSAP was shown to correlate with plaque macrophages and platelets, for which distribution in the plaque was uneven with high concentrations near the rupture site of the plaque. The association between presence of platelets and elevated FSAP expression in the symptomatic carotid plaques might be explained 3 different ways: (1) a large amount of platelets in the vulnerable vessel wall can excrete FSAP and/or attract such inflammatory cells as macrophages and activated monocytes, both expressing FSAP and leading to production of cytokines and chemokines, which in turn increase the expression of the FSAP¹⁷; (2) agglomerates of activated inflammatory cells release proteolytic enzymes and inflammatory cytokines, leading to cell apoptosis and necrosis of stabilizing cells, containing FSAP; or (3) FSAP, expressed on these cells, could be cleaved through the action of different enzymes, resulting in the increased release of activated FSAP.²⁹

We demonstrated that FSAP is expressed by isolated human peripheral platelets using immunologic approaches. Stimulation of purified platelets with ADP and TRAP increases expression of FSAP, indicating that FSAP expression is induced during platelets activation. Consequently, ADP is a suitable agonist that represents *in vivo* platelet stimulation and activation with increased FSAP expression, as indicated by real-time RT-PCR and immunofluorescence. However, TRAP and ADP are platelets activators known to modulate the expression of numerous genes in platelets and inflammatory genes like cytokines and chemokines, which have been implicated as important mediators of atherosclerosis.³⁰

Antiplatelet medication and carotid endarterectomy are effective for secondary stroke prevention.²⁷ Assuming aspirin or ASA as a cornerstone of antiplatelet therapy for coronary artery disease and

cerebrovascular disease, we decided to investigate the effect of ASA on platelet-related FSAP expression under ADP and TRAP stimulation. As expected, we observed decreased FSAP expression in activated platelets after treatment with ASA. The results provide evidence that FSAP expression within the hyperactivation of human platelets in the acute stage of cerebral ischemia could be limited on ASA treatment. Apart from its antithrombotic effect, ASA also manifests an anti-inflammatory action, exerted through mechanisms not completely clarified.³¹ Thus, it cannot be excluded that ASA, acting through complex interactions, indirectly reduces the proinflammatory function of blood platelets and the FSAP expression in the acute stage of atherothrombotic diseases. Despite the effective inhibition of COX1 (cyclooxygenase 1)-dependent platelet aggregation in patients with stroke taking ASA seem to be protected from the activity of proinflammatory mediators such as FSAP, released in excess by platelets. Further prospective studies in humans are needed to clarify the long-term effects of platelet-derived FSAP expression on efficacy of antiplatelet drugs in primary and secondary prophylaxis.

This study is the first to show that FSAP in human carotid plaques and plasma is associated with the presence of symptoms. In addition, this study shows that the levels of FSAP in plaque macrophages and platelets as its main source are also associated with the vulnerable plaque phenotype. We could show that FSAP is upregulated in platelets by stimulation with ADP and TRAP but decreases after treatment with ASA. Taken together, our data suggest that, under proatherogenic conditions such as inflammation, the different cell types present in atherosclerotic plaques respond by increasing their level of FSAP. Thus, FSAP could represent a new actor in the evolution of carotid atherosclerotic lesions, and it may represent new therapeutic targets for vascular proliferative diseases such as atherosclerosis.

Limitations

Our study has several limitations. The small number of study participants is a major limitation of our preliminary observational report. We did not routinely perform any measurements of CRP (C-reactive protein) or thrombin generation, so we are unable to explain the current findings with excessive systemic inflammation or enhanced thrombin generation after brain ischemia. However, we are able to rely on data in the literature to speculate on the possible impact of these phenomena in the context of our results. Furthermore, we did not perform any regression analysis of association between serum concentration of high-sensitivity CRP and FSAP in plasma from stroke patients, which might suggest that FSAP is an additional marker and/

or consequence of systemic inflammation. Moreover, this study was not designed to assess the association of the Marburg I variant with the survival of patients with aCAS and sCAS. Further epidemiological studies are required to elucidate whether carriers of the variant face a higher risk of thromboembolic events. In addition, we have not prospectively evaluated whether in general or at any moment after stroke ASA affects the studied platelet-derived FSAP expression in vivo. Further investigations are required to establish the pathway along which ASA exerts its effect on release of FSAP and, consequently, affects aggregation of platelets with monocytes. Moreover, we did not examine the dose-response effect on FSAP expression in platelets because data regarding platelet activation under different doses of ASA are conflicting. At the end, we did not calculate the values of plasma FSAP activity and concentration and examine the prognostic power of these at various time points after ischemic brain events with respect to the frequency of other cardiovascular clinical events, including nonfatal myocardial infarction, ischemic stroke, or death. However, our intention was not to examine the impact of special cardiovascular therapies on the levels of FSAP but rather to study the expression profile of FSAP in endarterectomy specimens that were taken from patients with asymptomatic and symptomatic carotid atherosclerotic plaques and to compare them with circulating FSAP levels. A multivariable analysis, the absence of which remains a limitation of the actual study, aiming to study the impact of variable therapies or situations, such as medication and smoking, is the goal of a future larger study from our group and is being planned.

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