The Prognostic Impact of Soluble Apoptosis-Stimulating Fragment on Mortality in Patients With Carotid Atherosclerosis

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Background and Purpose—Markers of apoptosis are associated with cardiovascular disease. The soluble apoptosis-stimulating fragment (sFAS) was found to be a predictor for outcome in patients with heart failure, but its importance in patients with atherosclerotic disease has not been fully understood as yet. The aim of the present study was to investigate the impact of sFAS on all-cause and cardiovascular mortality in patients with atherosclerosis in the carotid arteries.

Methods—We studied 981 of 1286 consecutive patients with neurological asymptomatic carotid atherosclerosis as evaluated by duplex Doppler sonography. Patients were prospectively followed for long-term all-cause and cardiovascular mortality.

Results—During a median follow-up of 6.2 years (interquartile range, 5.9 to 6.6 years), a total of 250 deaths (25.5%), including 165 (66%) cardiovascular deaths, were recorded. The risk for all-cause and for cardiovascular mortality, respectively, increased significantly with sFAS concentrations (P<0.001). The hazard ratio for all-cause death was elevated by 2.3-fold (P<0.001) and for cardiovascular death by 2.4-fold (P<0.001) in patients within the highest quintile of sFAS compared with patients within the lowest quintile, respectively. Results remained significant after adjustment for potential confounders and established cardiovascular risk factors, including high-sensitivity C-reactive protein. Patients with high sFAS but low high-sensitivity C-reactive protein had a comparable survival rate with those with elevated high-sensitivity C-reactive protein only (P=0.50).

Conclusions—Markers of apoptosis, as measured by sFAS, were found to be independent risk predictors for death in patients with atherosclerotic disease in the carotid arteries. (Stroke. 2011;42:2465-2470.)

Key Words: all-cause mortality ■ apoptosis ■ cardiovascular mortality ■ carotid atherosclerosis ■ soluble apoptosis-stimulating fragment

Atherosclerosis is one of the leading causes of morbidity and mortality in industrialized countries. The development of atherosclerotic lesions is a multifactorial and chronic process, including metabolic, inflammatory, hemodynamic, and genetic factors, leading to vascular remodeling and vessel damage. Apoptosis seems to be closely linked to the development of unstable atherosclerotic plaques. In this context, it has been demonstrated that markers of apoptosis are associated with clinical outcome in patients with cardiovascular disease. Recently, we demonstrated that proapoptotic molecules like the soluble apoptosis-stimulating fragment (sFAS) are independent risk predictors for outcome in patients with heart failure. However, the impact of sFAS on the development of atherosclerotic disease has not been fully understood as yet. Concentrations of sFAS were found to be associated with the development and presence of atherosclerosis in small cohorts with chronic renal disease. However, van der Meer and colleagues did not find sFAS to be associated with the presence of atherosclerosis determined by CT and ultrasonography in a community-based cohort. The importance of sFAS on long-term mortality among patients with atherosclerosis has also not been sufficiently investigated as yet.

So far, high-sensitivity C-reactive protein (hs-CRP) has been shown to be the most promising biomarker improving prediction of clinical events in patients with atherosclerosis. In particular, hs-CRP may help direct further evaluation and therapy in the primary prevention of cardiovascular disease.
disease in patients with intermediate cardiovascular risk. One small cross-sectional study indicated that sFAS may improve risk prediction in addition to hs-CRP. \textsuperscript{7}

We hypothesized that sFAS is associated with mortality in patients with atherosclerosis in the carotid arteries as evaluated by duplex Doppler sonography. We further assessed whether sFAS improves the risk prediction in addition to established cardiovascular risk factors and hs-CRP.

Methods

Study Design

The Inflammation in Carotid Arteries Risk for Artherosclerosis Study (ICARAS) is a prospective cohort study. Between March 2002 and March 2003, 1286 consecutive patients with carotid atherosclerosis but without recent transient ischemic attacks or strokes have been prospectively enrolled into ICARAS. The local ethics committee approved the study and all participants provided written informed consent.

Inclusion and Exclusion Criteria

Detailed information on inclusion and exclusion criteria is given elsewhere.\textsuperscript{11} In brief, patients with prevalent but clinical asymptomatic atherosclerosis of the carotid arteries, as evaluated by color-coded duplex Doppler sonography, were eligible. Patients with a history of a recent cardiovascular event (myocardial infarction, stroke, coronary revascularization, peripheral vascular surgery) during the preceding 6 months were not included. The rationale to exclude these patients was the assumption that acute cardiovascular events might impact sFAS levels and therefore might reflect the severity of an acute situation rather than chronic atherosclerotic disease.

Study End Point

All-cause mortality was defined as the primary study end point. As a secondary objective, we investigated the association between sFAS levels and cardiovascular mortality. The end points were evaluated by screening the national register of death, including screening for the specific cause of death (according to the International Statistical Classification of Diseases and Related Health Problems 10th Revisi-\textsuperscript{\textit{on}}). To prevent study participants being lost to follow-up due to migration or other causes, telephone contact (to the subject or relatives) was additionally established to check on the patients’ status. This was done if a patient was not seen at our outpatient department within the preceding 12 months. For the final analysis, no patient was lost to follow-up. The cause of mortality was confirmed in 43\% by postmortem examination.

Clinical Data

The patients’ medical history and clinical results from the physical examination were recorded at baseline. This included sex, age, height, weight, blood pressure, arterial hypertension, smoking status, diabetes mellitus, hyperlipidemia, family history of cardiovascular disease, history of cardiovascular events, and current medical treatment. The completeness of all demographics and vital parameters were checked for accuracy by 2 independent observers.

Laboratory Analysis

All standard laboratory analyses were determined according to local standard procedures. Serum sFAS was measured from frozen samples (−80°C) collected at the baseline study visit with the use of a quantitative sandwich enzyme immunoassay technique (R&D Systems, Minneapolis, MN). The assay range is 0.2 to 20.0 ng/mL; the intra-assay coefficient of variation ranges from 2.9\% to 4.6\%, and the interassay coefficient of variation ranges from 2.9\% to 6.7\%. For determination of hs-CRP, a high-sensitivity assay (N Latex CRP Mono; DADE Behring) with a detection level of 0.3 mg/L and a coefficient of variation of 4.6\% was used.

Statistical Methods

Continuous data are presented as the median and the interquartile range. Discrete data are given as counts and percentages. We used \textit{t} tests, \textit{x}\textsuperscript{2} tests, Mann-Whitney \textit{U} tests, Fisher exact tests, and Spearman correlation coefficients for univariate analyses, as appropriate. Time-dependent variables were analyzed using the Kaplan-Meier method and compared by the log-rank test. For this purpose, levels of sFAS were categorized in quintiles. Multivariate Cox proportional hazards models were applied to assess the association between sFAS and mortality. Results of the Cox models are presented as the hazards ratio and the 95\% confidence intervals (95\% CIs). To account for potential confounding effects, we calculated the risk for death by multivariate Cox proportional hazards analysis adjusting for age (years), sex (male/female), body mass index (kg/m\textsuperscript{2}), smoking (in categories), hypertension (binary), low-density lipoprotein cholesterol level (mg/dL), statin treatment (binary), glycohemoglobin A1 level (%), history of myocardial infarction (binary), peripheral artery disease (binary), history of stroke (binary), baseline degree of carotid stenosis (in categories), and hs-CRP (mg/L). We assessed the overall model fit using Cox-Snell residuals. Furthermore, we tested the proportional hazard assumption for all covariates using Schoenfeld residuals (overall test) and the scaled Schoenfeld residuals (variable-by-variable testing). According to the tests, the proportional hazards assumption was not violated. Receiver operating characteristic (ROC) curves were plotted to assess the contribution of sFAS to mortality risk in addition to that of established risk factors. ROC curves (deriving from the same cases) were compared as described by Hanley and colleagues.\textsuperscript{12} A 2-sided \textit{P}<0.05 was considered statistically significant. Calculations were performed with Stata (Release 8.0; StataCorp, College Station, TX) and SPSS for Windows (Version 15.0; SPSS Inc, Chicago, IL). Sample size calculation indicated that a sample size of 981 patients allows detecting a difference in mortality of 8\% assuming an overall mortality of 25\% during follow-up (\textit{p} 0.05, power 80\%).

Results

Patient Characteristics

A total of 1364 patients were screened during the initial study period. Of these, 78 subjects were not eligible because of recent cardiovascular events. A sensitivity analysis including these 78 patients revealed virtually identical effect sizes as the final analysis of the study population (data not shown). The remaining 1286 patients met eligible criteria and were enrolled in the study. From 305 patients, no frozen samples for determination of sFAS serum levels were available, leaving 981 patients for the final analysis. Of these, 617 patients (62.9\%) were male and the median age was 69 years (interquartile range, 61 to 76 years). Patients’ baseline characteristics and demographics are given in Table 1. There were no significant differences of baseline clinical characteristics (age, sex, frequency of atherothrombotic risk factors, and cardiovascular comorbidities) of the 305 patients who had to be excluded compared with the study population of 981 patients (data not shown).

Of 981 patients included in the final analysis, the association of sFAS at baseline and comorbidities is presented in Table 2. Age, male sex, hypertension, diabetes, and HbA1c were significantly associated with sFAS levels (Table 2). hs-CRP was not significantly associated with sFAS (\textit{r}=0.05, \textit{P}=0.137).

Outcome

During a median follow-up of 6.2 years (interquartile range, 5.9 to 6.6 years) corresponding to 5551 overall person-years,
a total of 250 deaths (25.5%) were recorded. Of these, 165 patients (66%) died of cardiovascular causes, 68 patients (27.2%) died of malignant diseases, 5 patients (2%) died of end-stage renal disease, and 12 patients (4.8%) died of other causes.

Univariate Analysis
A significant relationship between survival and sFAS levels was found. The risk for all-cause mortality increased significantly with concentrations of sFAS (P<0.001; Table 3) and was elevated by 2.3-fold (P<0.001) in the fifth quintile compared with the lowest quintile, respectively (Figure 1A). We additionally compared patients with sFAS in the fifth quintile (sFAS ≥13.25 ng/mL) with patients in quintiles 1 to 4. Using this cutoff, sFAS had good specificity (83%) but poor sensitivity (26%) for the prediction of death. The probability of death increased from 25% (pretest) to 33% (post-test) using this biomarker. Analyzing the risk for cardiovascular death revealed similar results as seen for all-cause mortality. The risk for cardiovascular death also increased significantly with sFAS concentrations (P<0.001; Table 3) and was elevated by 2.4-fold (P<0.001) in the fifth quintile compared with the lowest quintile, respectively (Figure 1B).

Multivariate Analysis and Analysis of Combined Strata
A likelihood ratio test indicated that the multivariate model including sFAS has a significantly better fit than the multivariate model including established atherosclerotic risk factors and hs-CRP only (P=0.018). In this multivariate model,
cardiovascular death was elevated by 1.6 ($P=0.067$) in the fifth quintile compared with the lowest quintile, respectively.

To evaluate a potential additive prognostic value of sFAS and hs-CRP based on their different pathophysiological associations, we assessed survival in combined strata of sFAS and hs-CRP. Patients with both markers below the median (Figure 2A) had a survival of 89% at 5 years. A survival of 83% and 81% at 5 years was observed in patients with either sFAS or hs-CRP above the median (log-rank test: $P=0.50$ between strata with 1 marker elevated; $P=0.058$ for high sFAS only versus no elevated marker; and $P=0.014$ for high hs-CRP only versus no elevated marker). Seventy-five percent of patients with sFAS and hs-CRP above the median survived for 5 years ($P=0.007$ versus high sFAS only, $P=0.04$ compared with high hs-CRP only). Similar predictive values of combined strata of sFAS and hs-CRP were found for the secondary end point cardiovascular mortality (Figure 2B). Patients with 1 of the 2 biomarkers elevated had a similar survival free from cardiovascular mortality ($P=0.74$). Patients with both biomarkers elevated had the highest rate of cardiovascular mortality.

C-Statistics

ROC curves were plotted to assess the diagnostic value for death within the median follow-up time of 6.2 years. The area under the ROC curve for isolated established risk factors was not significantly different from 0.5 (eg, 0.482 and 0.479 for total cholesterol and low-density lipoprotein cholesterol, respectively). The area under the ROC curve for a comprehensive score of 10 established risk factors (each of the
variables included in the multivariate model adds “1” to the score) was 0.618 (P < 0.001). Adding hs-CRP (<3 mg/L = 0; 3 to 10 mg/L = 1; >10 mg/L = 2) did not change the area under the ROC curve (0.618). Additional inclusion of sFAS concentrations (normalized to a scale from 1 to 10) increased the area under the ROC curve slightly to 0.63 (P < 0.001). The difference in the area under the curve between the ROC curve for a comprehensive score of 10 established risk factors and the ROC curve for the same risk score with additional inclusion of sFAS was not significant (P = 0.54).

Discussion
In the present study, sFAS was a significant predictor of all-cause mortality and cardiovascular mortality in a long-term follow-up of >6 years in patients with asymptomatic atherosclerotic disease in the carotid arteries. In accordance with previously published data about its role in heart failure, sFAS in the highest quintile equal to a concentration >13.25 ng/mL was associated with a >2-fold increase in risk. This pronounced increase of risk in the fifth quintile may indicate that sFAS concentrations are required to exceed a certain threshold to be linked to a poor clinical course. As previously reported, sFAS was associated with age, male sex, diabetes, and hypertension. Furthermore, sFAS was associated with long-term blood glucose control in our cohort. We could not confirm previously reported associations with obesity and smoking. Despite these associations with a number of important atherosclerotic risk factors, sFAS was an independent risk predictor and provided additive prognostic information. In summary, the present results in a large cohort of patients with carotid atherosclerosis extend the existing data about the role of sFAS in cardiovascular disease.

So far, our group has shown that sFAS is a prognostic factor for the outcome in patients with advanced heart failure. In patients with heart failure, sFAS was associated with a comparable increase in risk of death. sFAS may reflect increased apoptotic activity in the myocardium thereby increasing the progression of cardiomyopathy. Apart from its role in cell death in the myocardial tissue, sFAS may also destabilize atherosclerotic plaques in arteries by increased apoptosis of plaque-stabilizing cells such as endothelial cells and vascular smooth muscle cells forming the protective cap. sFAS mainly derives from the surface of activated immune cells such as cytotoxic lymphocytes and monocytes due to shedding. Plaque destabilization due to apoptosis of plaque-resident cells may ultimately lead to plaque rupture, the underlying cause for acute coronary syndrome. There is further evidence for the plaque-destabilizing effects of FAS. It has been shown to be present in advanced atherosclerotic plaques and overexpression of Fas/Fasl increases plaque vulnerability in pre-existing atherosclerotic lesions in an animal model. Furthermore, genetic data suggest that there is a causal association between sFAS and acute coronary syndrome. A single nucleotide polymorphism in the promoter region of FAS, which increases apoptosis of protective vascular smooth muscle cells, was associated with the occurrence of myocardial infarction. However, there are also some contradictory data that indicate that sFAS may also have protective effects.

Previous clinical studies have shown that sFAS concentrations are elevated in patients with acute coronary syndrome compared with healthy control subjects. Elevated sFAS increased the diagnostic accuracy for acute coronary syndrome. In contrast to troponin, sFAS concentrations were persistently elevated. However, sFAS was also elevated in patients with stable angina pectoris and concentrations were comparable to those of patients with myocardial infarction. These observational data do not allow differentiating whether sFAS is a cause or a consequence of plaque rupture leading to acute coronary syndrome. One smaller previous study did not find an association between sFAS and recurrent cardiac events. The present study extends the existing clinical data and demonstrates in a cohort study design that pre-existing sFAS concentrations during a stable phase of disease are predictive for acute cardiovascular events leading to death. Thus, sFAS may become a useful tool to improve risk prediction. sFAS in the fifth quintile (>13.25 ng/mL) may help to identify high-risk patients who might benefit from more intense control of common cardiovascular risk factors and more aggressive treatment. So far, we have shown in patients with heart failure that inhibitors of the renin–angiotensin–aldosterone system may potentially reduce sFAS concentrations. Future studies should clarify whether sFAS may improve the titration of plaque-stabilizing therapies such as statins. In contrast to the association of sFAS with acute events potentially induced by plaque rupture, we did not find an association of sFAS with the progression of atherosclerotic disease (data not shown). In accordance with this observation, a previous study has shown that sFAS is not associated with the extent of atherosclerosis.

Although this study shows that sFAS is an independent risk factor adding predictive value to a comprehensive risk prediction model for death during a long-term follow-up, the assessment of the diagnostic use of sFAS indicated that this apoptosis-related biomarker has only moderate diagnostic additive value, because the difference in the area under the curve between the ROC curve for a comprehensive score of 10 established risk factors and the ROC curve for the same risk score with additional inclusion of sFAS was not significant. However, this assessment does not take into account the time to event. The diagnostic value of sFAS is still better than that of separate established risk factors and comparable to a comprehensive score of established risk factors.

Limitations
We are aware of certain limitations of our study. The ICARAS population consists of preselected patients with known atherosclerosis and at high risk for cardiovascular events. In addition, the age range of our population is rather small, and younger individuals are underrepresented. Therefore, we cannot exclude an impact of the sFAS concentrations on survival in unselected subjects without prevalent atherosclerosis or without conventional risk factors. It may be of clinical interest to prospectively evaluate the impact of sFAS concentrations on the onset, progression, and outcome of cardiovascular disease in a community-based cohort without known atherosclerosis. However, the single-center and hospital-based design of our study facilitated patient moni-
toring and resulted in an excellent rate of follow-up. Potential intraindividual variations may be a limitation when determining sFAS only once in each patient. Repeated measurements of sFAS should be performed in future studies.

Conclusions

The apoptosis marker sFAS measured during a stable phase of atherosclerotic disease is an independent predictor of mortality. sFAS improves the prediction of outcome by established cardiovascular risk factors and hs-CRP. This association with the most important end point over a long-term follow-up of >6 years emphasizes its potentially important role in the identification of high-risk patients who may benefit from more aggressive treatment. The strong association of sFAS with mortality with a >2-fold increase of risk in the highest quintile may be explained by its persisting and stable elevation and its potentially important pathogenic role. Future studies are required to validate our results and to evaluate therapeutic modulation of sFAS concentrations and intraindividual changes over time in an independent cohort.

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Disclosures

None.

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### S1. Cox proportional hazard models for all-cause death according to quintiles of sFAS.

<table>
<thead>
<tr>
<th>Quintile</th>
<th>No. of subjects</th>
<th>No. of deaths</th>
<th>Person-years</th>
<th>HRs* (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st quintile</td>
<td>196</td>
<td>37</td>
<td>1200</td>
<td>1.0 (reference category)</td>
</tr>
<tr>
<td>2nd quintile</td>
<td>196</td>
<td>54</td>
<td>1117</td>
<td>1.6 (1.1-2.5)</td>
</tr>
<tr>
<td>3rd quintile</td>
<td>197</td>
<td>47</td>
<td>1132</td>
<td>1.4 (0.9-2.2)</td>
</tr>
<tr>
<td>4th quintile</td>
<td>196</td>
<td>47</td>
<td>1104</td>
<td>1.5 (0.9-2.3)</td>
</tr>
<tr>
<td>5th quintile</td>
<td>196</td>
<td>65</td>
<td>998</td>
<td>2.3 (1.5-3.4)</td>
</tr>
</tbody>
</table>

HR denotes hazard ratio, CI denotes confidence interval.

*p<0.001 for trend