Identification of Soluble Tumor Necrosis Factor-Like Weak Inducer of Apoptosis (sTWEAK) as a Possible Biomarker of Subclinical Atherosclerosis

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Objectives—Assessment of vascular risk in asymptomatic patients and the response to medical therapy is a major challenge for prevention of cardiovascular events. Our aim was to identify proteins differentially released by healthy versus atherosclerotic arterial walls, which could be found in plasma and serve as markers of atherosclerosis.

Methods and Results—We have analyzed supernatants obtained from cultured human carotid plaques and healthy arteries by surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry ProteinChip System. Surface-enhanced laser-desorption/ionization analysis unveiled an 18.4-kDa peak released in lower amount by carotid plaques than normal endarteries. This protein was identified as soluble tumor necrosis factor-like weak inducer of apoptosis (sTWEAK). To confirm that sTWEAK was the protein of interest, Western blot and enzyme-linked immunosorbent assay were performed. Both techniques confirmed that sTWEAK levels were decreased in carotid plaque supernatants. Subsequent measurement of sTWEAK in plasma showed a reduced concentration in subjects with carotid stenosis (N=30) compared with healthy subjects matched by sex and age (N=28) (P<0.001). Furthermore, in a test population of 106 asymptomatic subjects, we showed that sTWEAK concentrations negatively correlated with the carotid intima-media thickness (r=−0.4; P<0.001), an index of subclinical atherosclerosis.

Conclusions—These results suggest that sTWEAK could be a potential biomarker of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2007;27:916-922.)

Key Words: atherosclerosis ■ carotid arteries ■ plasma

Atherosclerotic plaque rupture is the trigger of cardiovascular complications such as myocardial infarction and stroke, which represent the first cause of death in Western countries. Atherogenesis is a complex process characterized by lipid deposition and a chronic inflammatory response. The resulting pathological vascular remodeling involves inflammatory cell recruitment, fibrosis, smooth muscle cell proliferation, and neovascularization. Our hypothesis is that atherosclerotic plaque prone to rupture could display a particular profile of released proteins, reflecting directly the late events preceding rupture such as proteolysis or cell death. The levels of several inflammatory molecules in circulating blood have been shown to be elevated in subjects at risk for an acute coronary event. Most of existing markers were proposed based on the assessment of proteins in plasma related to inflammation process associated with atherosclerosis (eg, C-reactive protein, CD40 ligand).

We have recently reported a new strategy to identify potential biological markers directly released by the arterial wall, using a proteomic approach. Incubation of endarterectomy samples versus control endarteries in a serum-free culture medium allowed us to harvest separately the proteins released from pathological and healthy areas and the supernatants (conditioned media) were subsequently analyzed by 2-dimensional electrophoresis. After identification of the differentially released proteins, their levels are measured in plasma to assess their potential as biomarkers of atherosclerosis. In this article, using a similar approach, we have analyzed the conditioned media from normal mammary endarteries versus carotid atherosclerotic endarterectomy samples by surface-enhanced laser desorption ionization time-of-flight (TOF) mass spectrometry (MS). This method is based on the chromatographic fractionation of the proteome on ProteinChips before MS analysis; it allows an easy and quantitative comparison of profiles of proteins released by atherosclerotic to those of control samples. Interestingly, the statistical validation of potential markers is performed before their identification. SELDI TOF enabled us...
Patients and Methods

Training Population (Patients With Carotid Atherosclerosis)

Thirty consecutive patients (carotid stenosis >70%; 22 men, 8 women; age, 62±9 years; 86% with hypertension, 39% with diabetes, 54% with hyperlipidemia) undergoing carotid endarterectomy at our institutions were included. Plasma samples were collected from patients before surgery and from 28 healthy volunteers (blood donors) matched for age (59±8) and sex (21 men, 7 women). Informed consent was obtained before enrolment. The study was approved by the local Ethical Committees in accordance with institutional guidelines.

Test Population (Asymptomatic Subjects)

The population studied consisted of 106 asymptomatic subjects (81 men, median age, 57 years [range, 25 to 78]) in whom global risk assessment was performed in the course of a general health check-up by Internal Medicine Department (University Clinic of Navarra, Spain). In all subjects, absence of history of coronary disease, stroke or peripheral arterial disease was recorded; additional exclusion criteria were the presence of severely impaired renal function, arthritis, connective tissue diseases, alcohol abuse, or use of nonsteroidal anti-inflammatory drugs in the 2 weeks before entering the study. The following conventional cardiovascular risk factors were defined as previously described:10,11 arterial hypertension and/or use of anti-hypertensive drugs; dyslipidemia and/or use of cholesterol-lowering drugs, obesity, smoking, and diabetes and/or use of pharmacological treatment. The local committee on human research approved the study, which was performed in accordance with the Declaration of Helsinki, and all participants gave written informed consent. In all subjects, carotid ultrasonography was performed to determine IMT, as previously described.9,11 Subjects were examined by the same 2 sonographers blinded to all clinical information. The reproducibility of IMT measurements between and within sonographers had previously been checked in 20 individuals who returned 2 weeks later for a second examination.9 The between-observer intra-class correlation coefficient was 0.76 (P<0.001) and the between-subject repeatability was 0.82 (P<0.001). The corresponding coefficients of variance were 5% and 10%, respectively.

Obtention of Endartery Conditioned Media

Carotid endarterectomy samples and mammary arteries were dissected and incubated as described previously.12 Conditioned media were collected and centrifuged, and protein concentration was determined by Bradford’s method. Tissue secretion attributed to necrosis during the incubation period was <10% as assessed by lactate dehydrogenase (LDH) release.7

SELDI TOF MS

Profiling of conditioned media from 7 mammary and 7 carotid atherosclerotic plaques (20 micrograms) was performed using various retention chromatography conditions: CM10 (cationic exchange array), Q10 (anionic exchange array), IMAC (immobilized metal affinity chromatography), and H50 (hydrophobic surface arrays). Samples were incubated in a Bioprocessor (Ciphergen), and the ProteinChip was then washed with the corresponding binding buffer and with ultrapure water. The arrays were allowed to air-dry and 0.6 mL of saturated solution of sinapinic acid in 50% v/v acetonitrile and 0.5% v/v trifluoroacetic acid was added twice to each spot. TOF of the retained proteins was measured using a ProteinChip reader (Ciphergen, PBS II). Validation was performed using this condition on 23 additional samples of control mammarys and atherosclerotic plaques. Calibration of SELDI-TOF was performed using all-in-one criteria, which are well suited for analysis of small (≤30 kDa) and potentially under-represented proteins. Among the differentially released proteins, we focused our attention on a 18.4-kDa protein released in larger amounts by controls relative to carotid plaques samples. This protein was identified as soluble tumor necrosis factor-like weak inducer of apoptosis (sTWEAK). More importantly, plasma levels of sTWEAK was measured in a training population of 30 patients with carotid atherosclerosis and 28 healthy subjects, showing the same trend as observed in conditioned media. Furthermore, sTWEAK was assessed in a test population consisting of 106 asymptomatic men in whom carotid artery intima/media thickness (IMT) was used as a marker of subclinical atherosclerosis.9

Figure 1. Protein profiles of secretomes from carotid plaques or mammary arteries. A, Mass spectra showing a global protein profile of conditioned media of 3 mammary (M1–M3) arteries versus 3 carotid (C1–C3) plaques on a CM10 ProteinChip pH5. The inset focuses on a peak of 18.4 kDa that is differentially released. B, SELDI quantification of 18.4-kDa protein secreted by mammary arteries vs. carotid plaques. Results are represented as mean±SD of log-normalized intensity.
peptide standard (C100–0005; Ciphergen) before measurements of all samples.

Isolation and Identification of the Marker of Interest

Mammary secretomes were pooled and subjected to weak cation exchange chromatography (CM10 spin columns; Ciphergen Biosystems) using 100 mmol/L ammonium acetate pH 5 as binding buffer. The same procedure was performed in parallel, using carotid-conditioned media containing nondetectable amounts of 18 400 m/z peak. Elution was performed by washing with increasing pH buffers (100 mmol/L Tris-HCl pH 7 to 10). The presence of the protein of interest was monitored on NP20 ProteinChip array and eluted at pH10. This elution fraction was concentrated and separated by SDS-PAGE (12% NuPAGE, Invitrogen). After silver staining, the band of interest (present in the pool of medium conditioned by mammary endarteries but not in the conditioned medium from complicated carotid plaques) was cut, destained, and dried. Gel pieces were covered with a 10 ng/mL sequencing grade trypsin (Sigma) in 50 mmol/L ammonium bicarbonate overnight at 37°C; 3 μL of the digestion solution plus 1 μL saturated CHCA (α-cyano-4-hydroxy-cinnamic acid) solution were added to H4 ProteinChip arrays. Mass spectra obtained by SELDI TOF MS were compared with those of blank gel pieces treated in the same conditions, and the peak masses only present in the sample were exported to Mascot with those of blank gel pieces treated in the same conditions, and the peak masses only present in the sample were exported to Mascot (http://www.matrixscience.com), using the National Center for Bio-technology Information and SwissProt databases.

Antigen–Antibody Capture

Analysis were performed on PS10 ProteinChips (Carbonyl Dilmidazole activated amine surface). Each spot was coated with 0.5 μg protein G (diluted in 50 mmol/L NaHCO3), blocked with 0.5 mol/L ethanolamine, and washed with phosphate-buffered saline containing 0.5% Triton X100 and 0.1% Triton X100. Then, 3 μL of goat anti-TWEAK (AF1090; R&D Systems) or goat IgG negative control antibody (AF1090; R&D Systems), anti-human CD68 (Dako).

Enzyme-Linked Immunosorbent Assay

Venous blood samples from different subjects were collected on EDTA. The whole-plasma samples were stored at −80°C until analysis was performed. Plasma concentrations of sTWEAK were determined in duplicate with commercially available enzyme-linked immunosorbent assay kits (BMS2006INST; Bender MedSystems). A total of 50 μL of plasma samples was assayed in parallel to known standard concentrations of recombinant TWEAK. The minimum detectable level of TWEAK was 10 pg/mL. Intra- and inter-assay coefficients of variation were 6.2% and 8.3%, respectively.

Immunohistochemistry

Specimens were fixed with paraformaldehyde and embedded in paraffin, and immunohistochemistry was performed on 4-μm-thick pieces as described previously11 using goat anti-TWEAK polyclonal antibody (AF1090; R&D Systems), anti-human α-actin (Dako), or anti-human CD68 (Dako).

Statistical Analysis

Statistical analysis was performed with SPSS for Windows software package version 11.0 (SPSS Inc, Chicago, Ill). Enzyme-linked immunosorbent assay data are expressed as medians and interquartile ranges and were analyzed by the Mann-Whitney U test. Univariate association was performed by Pearson correlation test. Multivariate linear regression analysis was conducted with carotid IMT as dependent variable, including in the model the traditional risk factors and those variables that were significant in the univariate analysis. A 2-tailed P<0.05 was considered statistically significant.

Results

In a first set of experiments, protein profiles were obtained for 7 mammary endartery-conditioned and 7 carotid-conditioned media using different ProteinChip chromatographic surfaces under various binding conditions. The most discriminating condition (CM10, weak cationic exchange surface, pH5) was used to test a total of 30 controls versus 30 carotid-conditioned media. Figure 1A shows 6 representative spectra obtained by analysis of conditioned media from mammary versus atherosclerotic carotid endarteries, focusing on a peak with a mass of 18.420 Da, which showed the highest statistical differences between the 2 conditions. The intensity of the 18.4-kDa peak, normalized to total ion current, was...
5-fold higher in mammary relative to carotid atherosclerotic conditioned medium (Figure 1B) (n=30, P<0.001). To identify the protein of interest, cationic exchange chromatography using CM10 spin columns in pH5 binding buffer was performed on a pool of mammary conditioned media and on conditioned medium from a carotid plaque, which did not contain the peak of interest. The 18.4-kDa peak was eluted by increasing the pH and monitored on NP20 ProteinChips. The fraction containing the protein of interest (pool of mammary conditioned media) and/or the similar fraction of carotid conditioned medium were concentrated (SpeedVac) and loaded onto a polyacrylamide gel. After comparison of the 2 profiles of proteins obtained on the silver-stained gel, the band corresponding to the 18.4 kDa peak, only present in the mammary samples, was cut and trypsin-digested. The products of trypsin digestion were analyzed using SELDI TOF MS, and the masses obtained for these peptides were exported to Mascot database (http://www.matrixscience.com) (Figure 2A). We found that the peptide masses obtained matched with those of the soluble form of TWEAK, digested in silico by trypsin, with a probability-based Mowse score of 76 (access number O43508). TWEAK also designated that Apo-3 Ligand has an expected molecular mass of 27.5 kDa; however, a soluble form of ≈18 kDa has been reported to be a secreted ligand belonging to the tumor necrosis factor superfamily.13 All matching peptides resulting from trypsin digestion covered the C-terminal region of TWEAK, corresponding to its soluble form. The overall sequence coverage attains 56% of the theoretical sequence of soluble TWEAK. These findings were consistent with the expected molecular mass of sTWEAK and a possible retention on a cationic exchange surface (at pH5, the protein is positively charged because its pI is 9.5). To confirm that the 18.4-kDa protein detected by the SELDI TOF is sTWEAK, capture experiments were performed on PS10 ProteinChips coated with protein G, using either goat anti-TWEAK or nonimmune goat IgGs. As depicted in the Figure 2B, the 18.4-kDa protein was captured by the anti-TWEAK antibody (directed against the extracellular domain of TWEAK) from the pool of mammary conditioned media, but not by the IgG control or in the carotid-conditioned medium.

To further confirm that sTWEAK is differentially released by control and atherosclerotic plaques, we have analyzed the secretomes of carotid endarterectomy samples versus control mammary arteries by Western blot and enzyme-linked immunosorbent assay using antibodies directed against the extracellular domain of TWEAK. Western blot analysis showed 2 different bands of 18 and 25 to 26 kDa, for which intensities were increased in control arteries relative to atherosclerotic plaques (Figure 3A). It is known that there is a consensus N-glycosylation site in soluble TWEAK at position 139 and that the glycosylated form of TWEAK has a molecular weight of ≈25 to 26 kDa.14 Incubation of mammary secretomes with PNGase F, which efficiently N-deglycosylates proteins, induces a diminution of the 25 to 26 kDa band, indicating that this band should be the glycosylated form of TWEAK (Figure 3B). Quantification of sTWEAK by enzyme-linked immunosorbent assay confirmed that atherosclerotic plaques released less sTWEAK than control mammary arteries (43.6 pg/mL [27.8 to 67.2] and
116.9 pg/mL [96.2 to 179.5], respectively) (Figure 3C). Furthermore, we assessed the presence of total TWEAK by immunohistochemistry in nonincubated tissues and showed that mammary arteries display a strong positivity for TWEAK relative to carotid plaques (not shown). Furthermore, TWEAK colocalizes with macrophages and smooth muscle cells in atherosclerotic plaques (Figure 3D).

To verify our hypothesis that plasma content can reflect arterial wall secretion, we have used a training set of plasma samples from patients with carotid atherosclerosis (stenosis >70%; n=30) and healthy subjects (n=28) matched by sex and age. We have observed that sTWEAK was diminished in atherosclerotic patients as compared with healthy subjects (202.6 pg/mL [148.1 to 239.9] versus 393.24 pg/mL [367.1 to 481.07], respectively; P<0.001) (Figure 4A).

To validate sTWEAK as a potential marker of atherosclerosis, it was assessed in plasma of a test population consisting of 106 asymptomatic subjects in whom IMT was measured. Characteristics of the studied population are summarized in Table 1. When we analyzed tertiles of sTWEAK there was a linear trend correlating the reduction of sTWEAK and the increase in carotid IMT (P<0.001). In addition, as shown in the Figure 4B, a univariate analysis shows an inverse correlation between sTWEAK and IMT (r=−0.402; P<0.001).

Furthermore, only marginal inverse correlations could be observed between sTWEAK and C-reactive protein (r=−0.18) or glucose concentrations (r=−0.19) (Table 2). Correlation between sTWEAK and IMT was superior to those observed between IMT and other clinical and laboratory parameters analyzed including C-reactive protein, a marker of systemic inflammation (Table 2). Interestingly, the association between sTWEAK and carotid IMT remained significant after adjusting for traditional risk factors and inflammatory markers (Table I, please see http://atvb.ahajournals.org).

Discussion

SELDI TOF MS is a recently described affinity-based MS method combining chromatography and MS. ProteinChip technology has proven to be useful in the discovery of potential diagnostic markers of different types of cancer, such as ovarian cancer. However, its use to discover new biomarkers related to atherosclerotic diseases has not been reported yet. We have previously validated an original approach analyzing the secreted proteomes from atherosclerotic plaques and nonpathological arterial wall for which incubation of the tissue in a serum-free medium allows the accumulation of proteins and their subsequent analysis without interference with plasma proteins. In the present study, to identify potential biomarkers reflecting the presence of complicated atherosclerotic plaques, protein profiling was performed on secretomes from endarterectomy samples compared with control endarteries by SELDI TOF MS. Although the use of healthy carotid arteries to compare with patholog-

<table>
<thead>
<tr>
<th>Table 1. Baseline Clinical Characteristics of the Studied Populations</th>
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<tbody>
<tr>
<td>Total Population (n=106)</td>
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<tr>
<td>Age, y</td>
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<tr>
<td>Sex, male/female</td>
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<td>Smokers, yes/no</td>
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<td>BMI, kg/m²</td>
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<td>SBP, mm Hg</td>
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<tr>
<td>HDL cholesterol, mg/dL</td>
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<tr>
<td>LDL cholesterol, mg/dL</td>
</tr>
<tr>
<td>Triglycerides, mg/mL</td>
</tr>
<tr>
<td>CRP*, mg/L</td>
</tr>
<tr>
<td>Fibrinogen, mg/dL</td>
</tr>
<tr>
<td>vWF, %</td>
</tr>
<tr>
<td>sTWEAK, pg/mL</td>
</tr>
<tr>
<td>Mean carotid IMT, mm</td>
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</tbody>
</table>

Values are expressed as mean±SD, N of subjects, and *mean±SEM. BMI indicates body mass index; CRP, C-reactive protein; DBP, diastolic blood pressure; IMT, intima-media thickness; SBP, systolic blood pressure; vWF, von Willebrand factor.

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The authors thank Sabine Jourdain (Ciphergen Inc) for her technical advices.
Sources of Funding
This study was supported by grants from Pfizer-Spain, Spanish Cardiovascular Network (RECAVA RD06/0014/0035), SAF 2004/06109, CAM (S2006/GEN-0247), Fondo de Investigaciones Sanitarias (CP04/00060; PI012047), Leducu Foundation, and AstraZeneca Future Forum (O.M. and J.B.M).

Disclosure
None.

References
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Arterioscler Thromb Vasc Biol. 2007;27:916-922; originally published online February 1, 2007; doi: 10.1161/01.ATV.0000258972.10109.ff

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Table I (online): Multiple linear regression analysis with carotid IMT as dependent variable.

<table>
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<th></th>
<th>B</th>
<th>SE (B)</th>
<th>Partial $R^2$ (%)</th>
<th>p</th>
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<td>IMT (mm) sTWEAK, pg/mL</td>
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<td>0.001</td>
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<td>Smoking</td>
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<td>0.041</td>
<td>30.3</td>
<td>0.003</td>
</tr>
</tbody>
</table>

B: regression coefficient; SE (B): standard error; $R^2$: partial correlation coefficient after adjustment. Adjusted for age, gender, smoking, BMI, glucose, total cholesterol, systolic blood pressure, fibrinogen, von Willebrand and CRP.